厚生労働科学研究費補助金
（成育疾患克服等次世代基盤研究事業）

女性生殖器における妊孕能の客観的な評価法の確立

平成20年度～22年度　総合研究報告書

主任研究者　吉野　修

平成23年3月

目　　次

課題番号: H20－子ども－若手－011

課題名:成育疾患克服等次世代基盤研究事業

「女性生殖器における妊孕能の客観的な評価法の確立」

主任研究者: 吉野 修 (東京大学 産婦人科)

研究総括

主任研究者: 吉野 修 (東京大学 産婦人科)

(要約) 不妊治療のニーズが高まる中、生殖機能の客観的な評価法が確立しているとは言い難く、このことが不妊治療の方針が定まらない原因と思われる。客観的なスクリーニング法を確立することが効率のよい治療法の提示および妊娠率向上に寄与すると思われる。特に機能評価が確立していない 1). 卵巣機能評価、2). 得られた卵子および胚の評価、3).子宮内膜の着床能の評価にわけ、各々の分野で精力的に基礎研究を行ってきた研究者と共同研究を行ってきた。全領域において基礎的な知見を得ることができた。また、子宮内膜の着床能評価に関して、MRI 法を用いた臨床データを蓄積することができた。

研究分担者

1. 卵巣機能

卵巣予備能の客観的評価に関する基礎的研究

福井大学 産婦人科 教授 小辻 文和

東京大学 産婦人科 特任研究員 吉野修

2．胚・卵子の客観的評価に関する研究

山形大学 工学部 教授 阿倍 宏之

3．着床に関する研究

a. 子宮内腔洗浄液を用いた子宮内膜の客観的評価に関する検討

慶應大学 産婦人科 講師 浜谷 敏生

b. 画像による子宮内膜の客観的評価法の開発

順天堂大学 放射線科 堀 正明

東京大学 産婦人科 特任研究員 吉野修

c. 子宮内膜評価 (腹腔内癒着に関する解析)

慶應大学 産婦人科 講師 浅田 弘法

d. 子宮内膜の客観的評価法の開発を目的とした胚着床に関する基礎的研究

東京大学 産婦人科 講師 大須賀 穣

研究目的

女性の加齢に伴い卵巣機能は低下し、子宮筋腫など子宮疾患の合併率が上昇することが知られている。女性の社会進出が進んでいる現代では女性の晩婚化が進んでおり、それに伴い卵巣および子宮機能異常に起因した不妊症患者が増えている。不妊治療を進める上で、正確な卵巣機能評価は治療方針の決定に大切である。また、近年体外受精のニーズは拡大しているが、得られた胚の評価方法も主観による形態的な評価にとどまっている。良好胚の正確な選別は妊娠率の向上のみならず、母子に対するリスクから産科領域で問題となっている多胎の予防にも寄与することから、新たな胚の評価方法が望まれる。また胚の子宮内膜への着床は未だ不明な点が多い。従来の不妊検査では異常が見つからない所謂、原因不明症例は不妊患者の4割を占めると言われている。この中に子宮内膜の機能異常症例が少なからず含まれていると思われるが、これまで子宮内膜機能に対する有効な検査法は全く確立していない。また、子宮筋腫合併症例などに対し、子宮病変の治療もしくは不妊治療のどちらを先行させるべきなのか明確な方針がない。

本研究では 1).卵巣機能評価方法 2).卵子・胚の評価 3).子宮内膜の評価にわけ、共同研究を通して新たな不妊症検査の確立をめざす。

精度の高い不妊スクリーニング検査の確立は、将来その概念に立脚した治療法の開発も多いに期待することができる。

A. 研究方法

1) 卵巣機能評価

（吉野担当）：卵胞発育のメカニズムを解明することが、正確な卵巣機能評価法の開発に有用である。近年、卵巣に発現するサイトカインとして、TGF-beta スーパーファミリーに属する bone morphogenetic protein (BMP)ファミリーが注目されている。ヒト卵巣由来の顆粒膜細胞を用い、BMPサイトカインの基礎的研究を網羅的におこなった。
また、ヒト卵巣の予備能力を測定するために同サイトカインファミリーの血中濃度の評価を行った。

(小辻担当)：BMP ファミリーサイトカインに属する GDF-9 の卵胞における働きを、甲状腺ホルモンとの関係を含めて基礎的検討した。

2) 胚および卵子の評価 （阿部担当）：胚や卵子の品質は胚移植による妊娠率に大きく影響することから、不妊治療における治療成績の向上には精度の高い生殖細胞の品質評価技術の開発が不可欠である。本研究では、電気化学計測技術を応用した非侵襲的呼吸測定装置により胚および卵子の呼吸能を解析し、呼吸活性を指標に妊娠が期待できる高品質胚の効率的選択法の開発を目的とする。電気化学計測技術と生物学的解析技術を駆使し、細胞呼吸活性を指標とする胚品質評価法の有効性と安全性を検証するとともに、ヒト胚（余剰胚）や卵子を用いた探索的臨床研究を実施した。

3) 子宮内膜評価：

1．子宮内腔洗浄液の検討（浜谷担当分）
今日の生殖医療において、良好な胚を胚移植しているにもかかわらず反復着床不全となる例に対して、治療法の開発は急務である。まず我々は、子宮内腔着床環境の評価法開発を

目指す。子宮内腔洗浄液中に存在するmatrix metalloproteinase (MMP)、CD9、HB-EGF などの内膜由来の液性因子に注目し、それらの分泌量(活性)と妊娠(着床)率との相関を検討することにより、着床因子のバイオマーカーとしての有用性を探索する。

2．画像による子宮内膜の客観的評価法の開発（堀担当分）他科領域で使用されている新たなMRI検査法を用い、不妊症例を対象に検討を行った。特に、子宮筋腫が子宮内膜に与える影響についてこれまで殆ど分かっていない。子宮内膜を動画MRI（Cine MRI）およびT２値測定という新しいMRIの手法を用いて評価した。

3．子宮内膜評価(腹腔内癒着に関する解析；浅田担当分)骨盤内癒着の存在は不妊の原因となりうる。また腹腔鏡手術を行う際にも手術操作を困難にする要因となる。したがって非侵襲的な骨盤内癒着の評価は不妊原因の診断および手術計画の面で有意義である。我々はCINE MRIによる骨盤内癒着の評価について検討を行った。FIESTA法によるCINE MRI撮影を行い、卵巣周囲およびダグラス窩周囲の癒着の評価を行った。

4．着床機転に関与する因子の基礎的研究（大須賀担当分）近年、胚の着床に炎症機転が作用することが知られており、サイトカインがその制御に関与することが知られている。ヒト子宮内膜の培養細胞を用い、種々のサイトカインが着床に向けてどのような作用を示すか網羅的に探索した。

C. 研究結果と　D. 考察

1). 卵巣機能評価

（吉野研究員担当）

我々は、まず基礎研究としてヒトにおけるBMPサイトカインに関する体系的、網羅的な検討を行い、BMP-2, -6, -7 のヒト卵巣において、FSH受容体を誘導することを見出した。また近年、ヒト卵巣機能の指標としてAMH(anti mullerian hormone)サイトカインが注目されている。我々はAMHがBMPサイトカインにより正に制御されていることを明らかにした。そして臨床研究ではヒト血液中のAMH, BMP サイトカインを測定することで、卵巣の生殖能力を示す指標に関して検討を行い、特にAMH 測定がヒト卵巣機能を評価する上で有用であるとの知見を得た。

(小辻教授担当)

上記 BMP サイトカインファミリーに属する GDF-9 は甲状腺ホルモンと相乗的に卵胞発育に寄与していることを見出した。甲状腺ホルモンは卵胞発育に重要であることがこれまで経験的に知られていたが、GDF-9 との作用を有することが明らかになった。

2). 胚・卵子の選別法

（阿部教授担当）

胚の新たな選別方法として、胚の酸素消費量測定を、走査型電気化学顕微鏡をベースに開発した「胚・卵子呼吸測定装置」を用いて調べた。マウスおよびヒトを用いた検討で、同法がミトコンドリアの機能を反映し胚の選別に有用であることを示すことができた。酸素消費量を測定す

ることで、胚の質を客観的に測定することができることを明らかにすることができ、現在、同法の実地臨床への応用を試みている。体外受精において、胚の選別方法は医療の効率化を図る上で、非常に重要な問題である。胚酸素消費量や胚の培養上清の検討により新たな胚選別の指標を打ち出すことが期待できる。

3).子宮内膜の評価法

（浜谷講師担当）

ヒト子宮内腔洗浄液と不妊症に関する検討において、特に MMP について測定し、子宮内腔洗浄液に MMP が強陽性の場合は抗生剤とプレドニゾロンによる内服療法による MMP のコントロールが奏功を示し、それが妊娠率の改善に導くことが明らかとなった。次に、子宮腔内の CD9 濃度と妊娠率との関係について検討を加えた。まず、マウスにおける基礎的検討を進めた結果、マウスでは CD9 が子宮内腔に分泌されていること、子宮内腔の CD9 濃度が低いと卵管に達する精子数が減少することが明らかとなった。さらに、ヒト子宮内腔洗浄液中の CD9 濃度をウェスタンブロット解析し、その後の妊娠予後を検討したところ、CD9 濃度は低い方が妊娠に有利であることが示唆された。

（大須賀講師担当）

ヒト細胞を用いた基礎的検討で、サイトカイン BMP-7 は子宮内膜の分化を制御することで胚の着床を促すこと、また胎児成分である絨毛細胞は低酸素環境により種々のサイトカイン、ケモカインを誘導することで、着床に寄与することを見出した。

（堀講師、吉野研究員担当）

我々は子宮筋腫が不妊症に与える影響について MRI を用いて検討を行った。動画ＭＲＩを用いた検討では、子宮筋腫により誘導される子宮内膜の異常蠕動運動が、妊娠を妨げる要因になっていることを示した。また、子宮筋腫の存在により、子宮内膜が炎症状態に陥っており、このことが妊娠の妨げになっていることを MRI によるＴ２値測定により示すことができた。

（浅田講師担当）

卵巣周囲癒着に関しては感度８０％、特異度８５％で癒着の有無を評価することが可能であった。一方、ダグラス窩周囲癒着に関しては感度８０％、特異度９３％で癒着の有無を診断することができた。CINE MRI は非侵襲的で撮影時間の延長があるものの患者に与える負担は軽微であり、骨盤内癒着を客観的に評価できる有用な検査であることがわかった。

近年、女性の晩婚化に伴い、不妊症患者に子宮内膜症による癒着や子宮筋腫を合併することが多く見受けられる。これら疾患を不妊治療の一環として手術すべきか否かは、医療の効率、医療費、患者負担の面からみて大変大きな問題となる。今回我々は画像検査により手術の要、不要症例を選びだすことの可能性を見出した。今後、同法が医療費の効率的使用に関与することが期待される。

F. 健康危険情報　　なし

G. 知的財産権の出願・登録状況

1. 特許取得（出願）

(1) 哺乳動物受精卵の呼吸活性測定方法及びその電気化学デバイス（珠玖仁、末永智一、高野真一郎、伊達安基、齋藤剛史、横尾正樹、伊藤隆広、阿部宏之）特願 2008-293117、2008 年 11 月.

（2）ウェルユニット及び電気化学的分析法（青柳重夫、内海陽介、末永智一、珠玖仁、阿部宏之、河野浩之、柏崎寿宣、星　宏良、星　翼）2010年9月17日、特願2010-208817号.

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
阿部宏之	ヒト卵・胚のクオリティー評価	森 崇英	卵子学	京都大学学術出版会		2009	
阿部宏之	胚の機能検定法	鈴木秋悦	カラーアトラス不妊治療のための卵子学	医歯薬出版		2010	127-131
阿部宏之	電気化学計測技術を応用したシングルセル呼吸機能解析と応用	神原秀記、松永是、植田充美	シングルセル解析の最前線	シーエムシー出版		2010	103-111

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Abe H., Hoshi H.	Morphometric and ultrastructural changes in ciliated cells of the oviductal epithelium in prolific Chinese Meishan and Large White pigs during the estrous cycle	Reproduction in Domestic Animals	Vol. 43	66-73	2008
那須 恵、熊迫陽子、後藤香里、宇津宮隆史、荒木康久、横尾正樹、阿部宏之	電気化学的呼吸能計測によるヒト胚のクオリティー評価	産婦人科の実際	Vol. 57, No. 2	289-294	2008
Utsunomiya T., Goto K., Nasu M., Kumasako Y., Araki Y., Yokoo M., Itoh-Sasaki T., Abe H.	Evaluating the quality of human embryos with a measurement of oxygen consumption by scanning electrochemical microscopy	Journal of Mammalian Ova Research	Vo. 25	2-7	2008

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shiku H., Yamakawa T., Nashimoto Y., Takahashi Y., Torisawa Y., Yasukawa T., Ito-Sasaki T., Yokoo M., Abe H., Kambara H., Matsue T..	A microfluidic dual capillary probe to collect messenger RNA from adherent cells and spheroids.	Anal.Biochem	385	138-142	2009
Murakawa H., Aono N., Tanaka T., Kikuchi H., Yoshida H., Yoshida H., Yokoo M., Abe H.	Morphological evaluation and measurement of the respiration activity of cumulus-oocyte complexes to assess oocyte quality.	J. Mamm. Ova Res.	26	32-41	2009
Kodama A, Yoshino O, Osuga Y et al.	Progesterone decreases bone morphogenetic protein (BMP) 7 expression and BMP7 inhibits decidualization and proliferation in endometrial stromal cells.	Hum Reprod.	25	751-6	2010
Takamura M, Koga K, Osuga Y, Takemura Y, Hamasaki K, Hirota Y, Yoshino O, Taketani Y.	Post-operative oral contraceptive use reduces the risk of ovarian endometrioma recurrence after laparoscopic excision.	Hum Reprod.	24	3042-8	2009
Shi J, Yoshino O, Osuga Y, Koga K, Hirota Y, Hirata T, Yano T, Nishii O, Taketani Y.	Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin beta subunits, and anti-Müllerian hormone in human granulosa cells.	Fertil Steril.	92	1794-8	2009
Shi J, Yoshino O, Osuga Y, Nishii O, Yano T, Taketani Y.	Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells.	Fertil Steril.	93	1273-9	2010
Orisaka M, Jiang JY, Orisaka S, Kotsuji F, Tsang BK.	Growth differentiation factor 9 promotes rat preantral follicle growth by up-regulating follicular androgen biosynthesis.	Endocrinology.	150	2740-8	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Orisaka M, Tajima K, Tsang BK, Kotsuji F.	Oocyte-granulosa-theca cell interactions during preantral follicular development.	J Ovarian Res.	2	9-	2009
Fukuda S, Orisaka M, Tajima K, Hattori K, Kotsuji F.	Luteinizing hormone-induced Akt phosphorylation and androgen production are modulated by MAP Kinase in bovine theca cells.	J Ovarian Res.	2	17-	2009
Kobayashi N, Orisaka M, Cao M, Kotsuji F, Leader A, Sakuragi N, Tsang BK.	Growth differentiation factor-9 mediates follicle-stimulating hormone-thyroid hormone interaction in the regulation of rat preantral follicular development model.	Endocrinology.	150	5566-74	2009
Abe H., Yokoo M., Itoh-Sasaki T., Nasu M., Goto K., Kumasako Y., Araki Y., Shiku H., Matsue T., Utsunomiya T.	Measurement of the respiratory activity of single human embryos by scanning electrochemical microscopy	Transactions of the Materials Research Society of Japan	Vol. 33, No. 3	759-762	2008
Yokoo M., Ito-Sasaki T., Shiku H., Matsue T., Abe H.	Multiple analysis of respiratory activity in the identical oocytes by applying scanning electrochemical microscopy	Transactions of the Materials Research Society of Japan	Vol. 33, No. 3	763-766	2008
Hirobe T., Ishizuka K., Ogawa S., Abe H.	Mitochondria are well developed in pink-eyed dilution melanoblasts in the neonatal mouse epidermis	Zoological Science	Vol. 25	1057-1065	2008
Toyoda M, Hamatani T, Matsumoto K, Saito H, Umezawa A.	Chip to analyze cell identity in Reproductive and Regenerative Medicine.	Current Medicinal Chemistry			In press

Yamada M, Hamatani T, Akutsu H, Chikazawa N, Kuji N, Yoshimura Y, Umezawa A.	Involvement of a novel preimplantation-specific gene encoding the high mobility group box protein Hmgpi in early embryonic development.	Human Molecular Genetics	19	480-493	2010
Wada-Hiraike O, Yamamoto N, Osuga Y, Yano T, Kozuma S, Taketani Y.	Aberrantimplantation and growth of uterine leiomyoma in the abdominal wall after laparoscopically assisted myomectomy.	Fertil Steril.	92	1747 e 13-5	2009
Koga K, Hiroi H, Osuga Y, Nagai M, Yano T, Taketani Y.	Autoamputated adnexa presents as a peritoneal loose body.	Fertil Steril.	93	967-8	2010
Koizumi M, Momoeda M, Hiroi H, Hosokawa Y, Tsutsumi R, Osuga Y, Yano T, Taketani Y.	Expression and regulation of cholesterol sulfotransferase (SULT2B1b) in human endometrium.	Fertil Steril.	93	1538-44	2010
Hasegawa A, Yoshino O, Osuga Y, Kodama A, Takamura M, Nishii O, Taketani Y.	Hyaluronic acid reagent suppressed endometriotic lesion formation in a mouse	Fertil Steril.	93	2757-9	2010
吉野　修、大須賀　穣	卵胞発育と血管新生	ホルモンフロンティア	17	227-235	2010
Kurotani R., Okumura S., Matsubara T., Yokoyama U., Buckley J.R., Tomita T, Kezuka K.	Secretoglobin 3A2 suppresses bleomycin-induced pulmonary fibrosis by TGFbeta signaling down-regulation.	J. Biol. Chem.			In press
Hirobe T., Yoshihara C., Takeuchi S., Wakamatsu K., Ito S., Abe H., Kawa Y. Soma Y.	A novel deletion mutation of mouse ruby-eye 2 named ru2d/Hps5ru2-d inhibits melanocyte differentiation and its impaired differentiation is rescued by L-tyrosine".	Zool. Sci.,			In press

Sugimura S., Yokoo M., Yamanaka K. Kawahara M., Wakai T., Nagai T., Abe H., Sato E.	Anomalous oxygen consumption in porcine somatic cell nuclear transfer embryos.	Cellular Reprogramming,	12	463-474	2010
Kimura N., Tsunoda T., Iuchi Y., Abe H., Totsukawa K., Fujiii J.	Intrinsic oxidative stress causes either two-cell arrest or cell death depending on developing stages of the embryos from SOD1-deficient mice.	Mol. Human Reprod.,	16	441-451	2010
後藤香里、小池恵、熊迫陽子、宇津宮隆史、荒木康久、阿部宏之	電気化学的呼吸計測技術におけるヒト胚クオリティー評価と安全性、	受精着床学会雑誌	27	53-8	2010
Sakagami N., Yamamoto T., Akiyama K., Nakazawa Y., Kojima N., Nishida K., Yokomizo S., Takagi Y., Abe H., Suzuki C., Yoshioka K	Viability of porcine embryos after vitrification using water-soluble pullulanfilms.	J. Reprod. Dev.	56	279-284	2010
Yamashiro H., Toyomizu M., Kikusato M., Toyama N., Sugimura S., Hoshino Y., Abe H., Moisyadi S., Sato E.	Lactate and adenosine triphosphate in extender enhance the cryosurvival of rat epididymal sperm.	J. Am. Assoc. Lab. Anim. Sci.,	49:	160-166.	(2010)
阿部宏之、吉田仁秋	電気化学計測技術を応用したヒト卵丘細胞−卵子複合体の呼吸能解析、	産婦人科の実際			、印刷中。
横尾正樹、伊藤-佐々木隆広、珠玖 仁、末永智一、阿部宏之	呼吸活性を指標とした胚の品質評価 −マウス胚移植試験の成績と産子の正常性について、	産婦人科の実際、	59	1375-1379	2010

後藤香里、小池 恵、熊迫陽子、宇津宮隆史、阿部宏之、	選択的単一胚移植(eSET)における移植胚選別困難例に対する呼吸量測定の有用性、	産婦人科の実際	59	1277-1281.	(2010)
Tsuji H, Miyoshi S, Ikegami Y, Hida N, Asada H, Togashi I, Suzuki J, Satake M, Nakamizo H, Tanaka M, Mori T, Segawa K, Nishiyama N,	Xenografted human amniotic membrane-derived mesenchymal stem cells are immunologically tolerated and transdifferentiated into cardiomyocytes.	Circ Res.	106	1613-23	2010
Masuda H, Matsuzaki Y, Hiratsu E, Ono M, Nagashima T, Kajitani T, Arase T, Oda H, Uchida H, Asada H, Ito M, Yoshimura Y, Maruyama T, Okano	Stem cell-like properties of the endometrial side population: implication in endometrial regeneration.	PLoS One.	28		2010
浅田弘法、古谷正敬、梶谷宇、内田浩、丸山哲夫、吉村泰典、升田博隆、岸郁子、田島敏秀、寺西貴英、木挽貢慈.	腹腔鏡下ダグラス窩子宮内膜症病巣切除術の定型化と疼痛緩和効果に関する検討.	日本エンドメトリオーシス会誌	31	39-43	2010;
岸郁子、浅田弘法、田島敏秀、木挽貢慈、河西朋代、小野寺成実、亀井清.	左水腎症にて判明した尿管子宮内膜症の1例.	日本エンドメトリオーシス会誌	31	146-150	2010
Yamada M, Hamatani T, Akutsu H, Chikazawa N, Kuji N, Yoshimura Y, Umezawa A.	Involvement of a novel preimplantation-specific gene encoding the high mobility group box protein Hmgpi in early embryonic development.	Hum Mol Genet.	19	480-93	2010.
Toyoda M, Hamatani T, Okada H, Matsumoto K, Saito H, Umezawa A.	Defining cell identity by comprehensive gene expression profiling.	Curr Med Chem.	17	3245-52	2010

Tarín JJ, Hamatani T, Cano A.	Acute stress may induce ovulation in women.	Reprod Biol Endocrinol.	26	53	2010.
Hamatani T.	Spermatozoal RNA profiling towards a clinical evaluation of sperm quality.	Reprod BioMed Online	22	103-105	2011
Nishijima K, Takahashi J, Yamamoto M,	Images in emergency medicine. Pregnant woman with gastri	Ann Emerg Med.	55	483	2010
Hirota Y, Osuga Y, Hasegawa A, Kodama A, Tajima T, Hamasaki K, Koga K, Yoshino O, Hirata T, Harada M, Takemura Y,	Interleukin (IL)-1beta stimulates migration and survival of first-trimester villous cytotrophoblast cells through endometrial epithelial cell-derived IL-8.	Endocrinology	150	350-6	2009
Hirota Y, Acar N, Tranguch S, Burnum KE, Xie H, Kodama A, Osuga Y, Ustunel I, Daikoku T, Dey SK.	Uterine FK506-binding protein52(FKBP52)-peroxiredoxin-6 (PRDX6) signaling protects pregnancy from overt oxidative stress.	Proc Natl Acad Sci U S A.	107	15577-82	2010
Mori M, Kitazume M, Ose R, Kurokawa J, Koga K, Osuga Y, Arai S, Miyazaki T.	Death effector domain-containing protein (DEDD) is required for uterine decidualization during early pregnancy in mice.	J Clin Invest.	121	318-27	2010

Growth Differentiation Factor 9 Promotes Rat Preantral Follicle Growth by Up-Regulating Follicular Androgen Biosynthesis

Makoto Orisaka, Jin-Yi Jiang, Sanae Orisaka, Fumikazu Kotsuji, and Benjamin K. Tsang

Reproductive Biology Unit and Division of Reproductive Medicine (M.O., J.-Y.J., S.O., B.K.T.), Departments of Obstetrics and Gynaecology and Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8L6; Chronic Disease Program, Ottawa Health Research Institute, The Ottawa Hospital (Civic Campus), Ottawa, Ontario, Canada K1Y 4E9; and Department of Obstetrics and Gynecology (M.O., S.O., F.K.), University of Fukui, Matsuoka, Fukui, Japan 910-1193

The transition from preantral to early antral stage is the penultimate stage of ovarian follicular development in terms of gonadotropin dependence and follicle destiny. Although oocyte-somatic cell communication is important in early follicular development, our knowledge of the precise role of the oocyte-derived growth differentiation factor (GDF)-9 during preantral follicle growth is incomplete. We examined whether and by what means oocyte-derived GDF-9 controls follicular development and steroidogenesis during the preantral to early antral transition, by a combination of *in vitro* gene manipulation (*i.e.* intraoocyte injection of GDF-9 antisense oligos) and preantral follicle culture. Intraoocyte injection of GDF-9 antisense suppressed rat preantral follicle growth *in vitro*, whereas GDF-9 enhanced follicular development. GDF-9 augmented testosterone production in preantral follicles. GDF-9 antisense suppressed androgen production and CYP17A1 mRNA expression in cultured follicles, a response attenuated by exogenous GDF-9. The nonaromatizable androgen 5α-dihydrotestosterone rescued the follicular growth arrest caused by GDF-9 down-regulation. The specific androgen receptor antagonist flutamide suppressed GDF-9-induced preantral follicle growth *in vitro*. The data suggest that GDF-9 plays an important role in promoting preantral follicle growth by up-regulating follicular androgen biosynthesis. GDF-9 is essential for CYP17A1 expression during follicular development from the preantral to the early antral stage. (*Endocrinology* 150: 2740–2748, 2009)

The ovarian follicle, consisting of an oocyte surrounded by granulosa and thecal cells, represents the basic functional unit of the ovary. Follicular growth can be classified into three phases according to their developmental stage and gonadotropin dependence (1): 1) follicular growth through primordial, primary, and secondary stages (gonadotropin independent phase); 2) transition from preantral to early antral stage (gonadotropin responsive phase), and the growth of these follicles is primarily controlled by intraovarian regulators and does not require gonadotropins (2), although it is also stimulated by the presence of FSH (1); and 3) continual growth beyond the early antral stage (gonadotropin dependent phase), which includes follicle recruitment, selection, and ovulation (3). The preantral-early antral transition is the penultimate stage of development in terms of gonadotropin dependence and follicle destiny [growth *vs.* atresia (4)].

Oocyte-somatic cell communication plays a critical role in folliculogenesis, including activation of resting follicles, early growth, and terminal differentiation (5, 6). Growth differentiation factor (GDF)-9 is an oocyte-derived factor and a member of the TGF-β superfamily, which includes TGF-β, activin, and bone morphogenetic proteins (BMPs) (7, 8). GDF-9 is expressed in the mammalian oocyte throughout follicular development (9, 10). Deletion of the GDF-9 gene in mice blocked folliculogenesis at the primary stage, demonstrating the importance of this growth factor in early follicular development (11). GDF-9 stimulates granulosa cell mitosis (12) and suppresses granulosa cell apoptosis (4) in rat small antral follicles. GDF-9 has been shown to

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2008-1536 Received November 5, 2008. Accepted January 30, 2009.
First Published Online February 12, 2009

Abbreviations: AR, Androgen receptor; BMP, bone morphogenetic protein; CTL, control; DHT, 5α-dihydrotestosterone; EIA, enzyme immunoassay; GDF, growth differentiation factor; MO, morpholino antisense oligonucleotide; Smad, Sma- and Mad-related protein.

enhance rat preantral follicle growth (13), although its precise mechanism(s) is obscure.

Ovarian steroids, which include progesterone, androgen, and estrogen, act via specific nuclear receptors and are essential for normal folliculogenesis and ovulation (14). Progesterone receptor (15) or estrogen receptor (16) null mice are infertile, and androgen receptor (AR) null mice culminate in reduced fertility and premature ovarian failure (17), indicating that these steroids are essential for reproductive function and fertility. Although the exact role of GDF-9 on follicular cell differentiation during the transition of the follicle from preantral to early antral stage is not clear, GDF-9 is known to stimulate basal estradiol synthesis and suppress FSH-induced progesterone and estradiol production in undifferentiated rat granulosa cells (12). The role of GDF-9 on thecal cell androgen production is less clear. Whereas Solovyeva *et al.* (18) showed that GDF-9 stimulates androstenedione production in rat theca-interstitial cell, Spicer *et al.* (19) reported that GDF-9 inhibits androstenedione production by bovine thecal cells from small antral follicles. Although GDF-9 promotes granulosa cell mitosis and preantral follicle growth, whether the latter response is mediated via follicular steroidogenesis is not known.

In the present studies, we hypothesized that oocyte-derived GDF-9 stimulates preantral follicle growth in part by up-regulating follicular steroidogenesis. We examined whether and by what means GDF-9 and FSH regulate follicular development and steroid production during preantral-early antral transition by a combination of *in vitro* gene manipulation and preantral follicle culture. We have demonstrated that GDF-9 promotes preantral follicle growth by stimulating follicular androgen biosynthesis.

Materials and Methods

Materials

All culture media and supplements were purchased from Life Technologies Inc. (Burlington, Ontario, Canada). Bovine insulin, human transferrin, ascorbic acid, sodium selenite anhydrous, L-glutamine, and agarose (low gelling temperature) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human FSH was obtained from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA). Morpholino antisense oligos (MOs) for control (CTL) and GDF-9 were purchased from Gene-Tools, LLC (Philomath, OR). Intracytoplasmic sperm injection micropipettes (no. MIC-35-30) were from Humagen (Charlottesville, VA). Goat antihuman GDF-9 antibody (C-18) and its blocking peptide as well as goat ImmunoCruz staining system were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Progesterone and testosterone enzyme immunoassay (EIA) kit were from R&D Systems, Inc. (Minneapolis, MN), whereas the estradiol EIA kit was from Calbiotech, Inc. (Spring Valley, CA). 5α-Dihydrotestosterone (DHT), estradiol, and flutamide, a specific AR antagonist, were from Sigma-Aldrich Co. (St. Louis, MO). RNeasy microkit and QuantiTect SYBR Green PCR kit were purchased from QIAGEN, Inc. (Mississauga, Ontario, Canada). Random decamer primers were from Ambion, Inc. (Austin, TX). PCR primers for CYP17A1, AR, and 18S rRNA were from Invitrogen Canada, Inc. (Burlington, Ontario, Canada). Recombinant rat GDF-9 was generously provided by Dr. Aaron J. W. Hsueh (Stanford University School of Medicine, Stanford, CA).

Preantral follicle isolation and culture

All animal procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Ottawa Health Research Institute Animal Care Committee. Female Sprague Dawley rats were obtained from Charles River Canada (Montréal, Québec, Canada) and maintained under standard conditions.

Large preantral follicles (diameter, 150–170 μM) were isolated from 14-d-old rats in Leibowitz L-15 medium with BSA (0.1%, wt/vol) at d 0, using 28.5-gauge needles (Becton Dickinson and Co., Franklin Lakes, NJ). Only round follicles with intact basement membrane and thecal layer were selected for the present studies. Follicles were cultured individually in a 96-well plate (Sarstedt, Inc., Newton, NC; no. 83.1837.50) in 100 μl of α-MEM supplemented with HEPES (10 mM), BSA (0.1%, wt/vol), bovine insulin (5 μg/ml), transferrin (2 μg/ml), ascorbic acid (25 μg/ml), sodium selenite anhydrous (2 ng/ml), L-glutamine (3 mM), sodium pyruvate (100 μg/ml), streptomycin (100 μg/ml), and penicillin (100 U/ml), with or without 100 ng/ml of GDF-9 and different concentrations of FSH (10 or 100 ng/ml). Preliminary data indicated that 100 ng/ml of GDF-9 or 10 ng/ml of FSH are the minimal effective concentrations for inducing a significant increase in preantral follicle growth in our culture system. Follicular diameter was measured daily as the average of distance between the outer edges of the basement membrane in two perpendicular planes and results were expressed as change in follicular volume. The percentage change of follicular volume on day n of culture is defined as the volume difference between day n and d 0 (the day of isolation) expressed as a percentage of the volume at d 0. The culture medium was changed every other day, and the spent media were kept at −20 C for steroid assays.

Intraoocyte injection of MO oligonucleotides in preantral follicles

Expression of MOs is effective in suppressing translation of target genes in zebrafish (20) and *Xenopus* (21) embryos. As described previously (4), to assess the role of GDF-9 on follicular development and steroidogenesis during preantral-early antral transition, GDF-9 content in the cultured follicles was manipulated by intraoocyte microinjection of GDF-9 MO. The GDF-9 MO sequence was designed based on its rat cDNA sequence (5′-ACAGGAATCTGCTGGGAAATGCCAT-3′, not homologous with BMP-15/GDF-9B cDNA). The standard CTL MO sequence (5′-CCTCTTACCTCAGTTACAATTTATA-3′) designed by Gene-Tools was not expressed in the follicles and caused no phenotype on follicular growth and steroidogenesis in the injected/cultured preantral follicles. The MOs were fluorescent tagged with Lissamine, which was visible by fluorescence microscopy. Large preantral follicles isolated from 14-d-old rats were cultured individually in a 96-well plate without GDF-9 or FSH. After 16–24 h, only the follicles (diameter 150–170 μM) with intact basement membrane and thecal layer were selected for the microinjection. CTL MO or GDF-9 MO (10 μM) was injected into the oocyte of the preantral follicles at d 0. The volume of MO injected (3 pl) was less than 5% (vol/vol) of the oocyte volume. Successful injection was confirmed by visualization of fluorescence (Lissamine tag). At d 1 (*i.e.* 24 h after intraoocyte injection), the oocyte morphology was evaluated microscopically, and only follicles with morphologically intact oocytes were cultured with or without GDF-9 (100 ng/ml) and FSH (10 ng/ml) for another 3 d (d 1 to d 4). Follicular diameter was measured daily and the culture medium was changed every other day. The percentage change of follicular volume on day n of culture is defined as the volume difference between day n and d 0 (the day of microinjection) expressed as a percentage of the volume at d 0. At the end of the culture period, follicles were fixed and embedded, as described previously (7), for GDF-9 immunohistochemistry. The spent media were kept at −20 C for steroid assays. The follicle were also pooled and kept at −80 C for real-time PCR analyses.

To assess the role of androgen and estrogen on GDF-9-induced preantral follicle growth, the CTL MO- and GDF-9 MO-injected (at d 0) follicles were cultured with different concentrations of DHT (0 to 10 μM) or estradiol (0 to 10 μM) for 3 d (d 1 to d 4). Moreover, to ascertain the

direct effect of androgens, the CTL MO- and GDF-9 MO-injected follicles were preincubated for 1 h (at d 1) with different concentration of the AR antagonist flutamide (0–10 μM) before the addition of GDF-9 (100 ng/ml) and cultured for another 3 d (d 1 to d 4). Follicular diameter was measured daily, and results were expressed as change in follicular volume.

GDF-9 immunohistochemistry

The GDF-9 content in the injected/cultured follicles was examined by immunohistochemistry, according to the previous protocol (7). The intensity of GDF-9 immunostain in 10 oocytes for each group at d 4 of culture was semiquantified using a relative scale: 0, 1, and 2 for no (see Fig. 2Ad), weak, and strong (see Fig. 2Ac) staining, respectively.

Steroid assays

The levels of progesterone, testosterone, and estradiol in the spent media were measured using the respective EIA kits (as described in *Materials and Methods*) according to the manufacturer's instructions. The intraassay coefficient of variation for progesterone, testosterone, and estradiol were 6.0, 9.5, and 10.9%, respectively, whereas the interassay coefficient of variation for progesterone, testosterone, and estradiol was 5.9, 11.7, and 12.5%, respectively. The sensitivity of the progesterone, testosterone, and estradiol assays was 9, 6, and 10 pg/ml, respectively.

Real-time PCR analysis of CYP17A1 and AR expression

Total RNAs from three MO-injected follicles (pooled from the same experiment group) were extracted, using RNeasy microkit according to manufacturer's instructions. Real-time quantitative PCR analysis for CYP17A1 and AR was performed on the follicular cDNAs, using a LightCycler 2.0 system (Roche Diagnostic Canada, Laval, Quebec, Canada). The primers of CYP17A1 used for amplification were a 5′-forward primer (5′-ACTGAGGGTATCGTGGATGC-3′) and a 3′-reverse primer (5′-TC GAACTTCTCCCTGCACTT-3′), whereas those of AR were a 5′-forward primer (5′-GGGTGACTTCTCTGCCTCTG-3′) and a 3′-reverse primer (5′-AAACGTGGTCCCTGGTACTG-3′). The transcript levels of CYP17A1 and AR were normalized against those of 18S rRNA (5′-forward primer; 5′-CGCGGTTCTATTTTGTTGGT-3′, 3′-reverse primer; 5′-AGTCGGCATCGTTTATGGTC-3′). Amplification reaction was then performed using the QuantiTect SYBR Green PCR kit (QIAGEN). The thermal cycling conditions comprised an initial denaturation step at 95 C for 15 min and 50 cycles (CYP17A1) or 40 cycles (AR and 18S rRNA) at 95 C for 15 sec, 56 C for 20 sec, and 72 C for 30 sec. The levels of CYP17A1 and AR mRNA were expressed as a ratio to 18S rRNA values.

Statistical analysis

Results are presented as means ± SEM of at least three independent experiments. All data were subjected to one- or two-way (repeated measure) ANOVA, except unpaired *t* test for immunostaining intensity of GDF-9 in oocytes (Prism 4.0 and InStat 3.0 statistical software; GraphPad Software, Inc., San Diego, CA). Differences between experimental groups were determined by the Tukey or Bonferroni posttest. Statistical significance was inferred at $P < 0.05$.

Results

Effects of GDF-9 and FSH on preantral follicular growth and steroidogenesis *in vitro*

To examine the effect of GDF-9 and FSH on preantral follicular growth, rat large preantral follicles were cultured with or without 100 ng/ml of GDF-9 and different concentrations of FSH (10 or 100 ng/ml) for 4 d. Preantral follicles cultured in the absence of GDF-9 and FSH exhibited minimal growth (Fig. 1A).

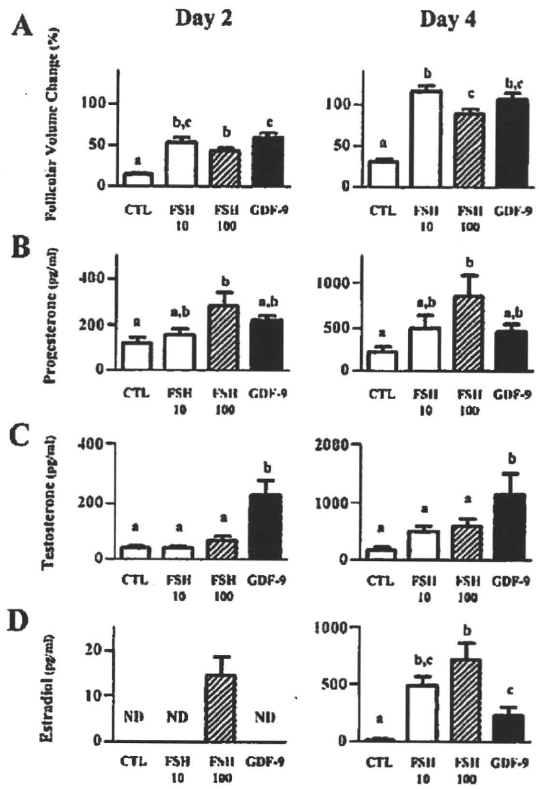

FIG. 1. Effects of GDF-9 and FSH on preantral follicular growth and steroidogenesis *in vitro*. A, Rat preantral follicles (150–170 μM in diameter) were cultured for 4 d with or without GDF-9 (100 ng/ml) and different concentrations of FSH [10 ng/ml (FSH_{10}) or 100 ng/ml (FSH_{100})]. CTL, Preantral follicles were cultured in the absence of GDF-9 and FSH. Follicular diameter was measured daily and results were expressed as change in follicular volume. The percentage change of follicular volume on day n of culture is defined as the volume difference between day n and d 0 (the day of isolation) expressed as a percentage of the volume at d 0. Results are presented as means ± SEM of a total of 40 follicles from six to eight independent experiments. ND, Not detected. *Bars with different superscripts* are significantly different at $P < 0.05$. Note that GDF-9 and FSH stimulated preantral follicular growth (A). B–D, Concentrations of progesterone (B), testosterone (C), and estradiol (D) in the spent media on d 2 and d 4 were measured. Note that GDF-9 augmented testosterone and estradiol production in preantral follicles, whereas FSH stimulated progesterone and estradiol production in the follicles.

An increase in follicular volume was seen as early as 2 d after treatment with either GDF-9 or FSH ($P < 0.05$ *vs.* CTL). Addition of GDF-9 to the culture media significantly increased follicular growth at d 4 ($P < 0.01$ *vs.* CTL; Fig. 1A). Because10 ng/ml of FSH appeared more effective on preantral follicle growth than 100 ng/ml of FSH at d 4 ($P < 0.01$), 10 ng/ml of FSH was used for the later microinjection studies.

To determine the influence of GDF-9 and FSH on follicular steroidogenesis during preantral follicle growth, the concentrations of progesterone, testosterone, and estradiol in the spent media were determined. Although 100 ng/ml of FSH augmented progesterone production in preantral follicles ($P < 0.05$ *vs.* CTL), neither 10 ng/ml of FSH nor GDF-9 significantly influences progesterone biosynthesis (Fig. 1B). Treatment with GDF-9 stimulated testosterone production in the preantral follicles ($P < 0.01$ *vs.* CTL), whereas FSH did not significantly affect testosterone level in the follicles (Fig. 1C). GDF-9 and FSH augmented estrogen production in the preantral follicles. We found

100 ng/ml of FSH to be more effective in stimulating estradiol production than GDF-9 in the follicles at d 4 ($P < 0.01$), although no significant difference was observed between 10 ng/ml of FSH and GDF-9 treatment group (Fig. 1D). It appeared that GDF-9 augmented testosterone and estradiol production in preantral follicles, whereas FSH stimulated progesterone and estradiol production in the follicles.

Effect of intraoocyte injection of GDF-9 MO on preantral follicle growth *in vitro*

To assess the role of GDF-9 on follicular development and steroidogenesis during the preantral to early antral transition, GDF-9 MO or its control MO (CTL MO) was injected into the oocyte of cultured preantral follicles (Fig. 2Aa). Successful injection was confirmed by visualization of fluorescence (Fig. 2Ab). The relative intensity of GDF-9 immunostain in GDF-9 MO group (0.33 ± 0.21; Fig 2Ad) was significantly lower than those of CTL MO group (1.67 ± 0.21; Fig. 2Ac), indicating that GDF-9 MO markedly decreased oocyte GDF-9 content *in vitro* ($P < 0.01$).

In the absence of GDF-9 and FSH, the CTL MO-injected preantral follicles exhibited minimal growth [follicular volume change at d 4: 60.4 ± 7.8% (CTL MO + CTL); Fig. 2Ba, C, and D]. Addition of GDF-9 (100 ng/ml) to the culture media significantly increased the follicular growth (CTL MO + GDF-9; Fig. 2Bb), and the increase at d 4 was 129.2 ± 13.0% ($P < 0.01$ *vs.* CTL MO + CTL; Fig. 2, C and D). Intraoocyte injection of GDF-9 MO suppressed basal preantral follicle growth during 4-d culture period (Fig. 2Bc). Whereas basal follicular volume in the CTL MO + CTL group was significantly increased by d 4 ($P < 0.05$), a decrease was noted in the GDF-9 MO + CTL group (−19.8 ± 5.1%), resulting in a significant difference between the two experimental groups ($P < 0.01$; Fig. 2, C and D). The effect of GDF-9 MO appeared to be specific to GDF-9 because the addition of GDF-9 to the culture media prevented the follicular growth arrest caused by GDF-9 MO (Fig. 2Bd). No significant difference in the change in follicular volume was observed between the CTL MO + CTL and the GDF-9 MO + GDF-9 group ($P > 0.05$; Fig. 2, C and D).

FSH (10 ng/ml) stimulated preantral follicle growth ($P < 0.05$, CTL MO + FSH *vs.* CTL MO + CTL; Fig. 2D). In the presence of FSH, GDF-9 down-regulation also suppressed follicular growth ($P < 0.01$, GDF-9 MO + FSH *vs.* CTL MO + FSH).

Effect of intraoocyte injection of GDF-9 MO on steroidogenesis in preantral follicles *in vitro*

Although FSH augmented progesterone production in preantral follicles ($P < 0.01$, CTL MO + FSH *vs.* CTL MO + CTL), neither the addition of GDF-9 nor GDF-9 down-regulation significantly influence progesterone biosynthesis irrespective of the presence of FSH (Fig. 3A). There was no significant difference in the progesterone concentration between the CTL MO + CTL, CTL MO + GDF-9, GDF-9 MO + CTL, and GDF-9 MO + GDF-9 group ($P > 0.05$).

Treatment with GDF-9 stimulated testosterone production in the follicles injected with CTL MO [550.3 ± 108.5 pg/ml (CTL MO + GDF-9) *vs.* 268.6 ± 78.0 pg/ml (CTL MO + CTL), $P <$ 0.05; . 3B]. GDF-9 MO markedly decreased androgen biosynthesis [14.1 ± 5.7 pg/ml (GDF-9 MO + CTL), $P < 0.05$ *vs.* CTL MO + CTL], a response attenuated by exogenous GDF-9 [475.9 ± 110.5 pg/ml (GDF-9 MO + GDF-9), $P < 0.01$ *vs.* GDF-9 MO + CTL]. The amount of testosterone in GDF-9 MO + GDF-9 was not different from those in CTL MO + CTL ($P >$ 0.05; Fig. 3B). FSH did not affect testosterone level in the CTL

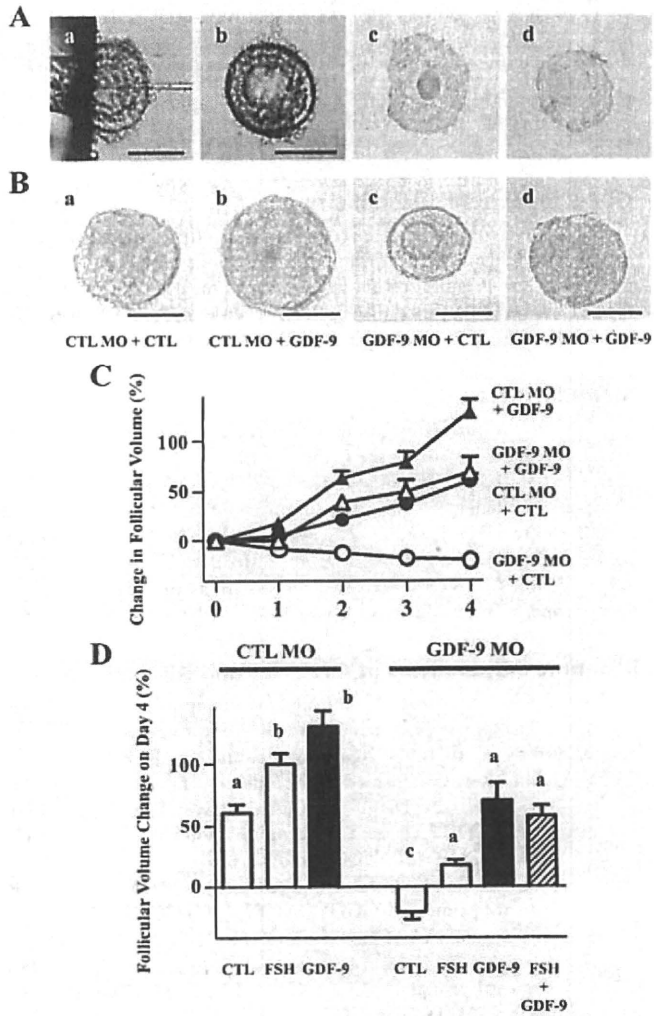

FIG. 2. Intraoocyte injection of CTL/GDF-9 MO in cultured preantral follicles and the influence of GDF-9 down-regulation on follicular growth *in vitro*. A, a and b, Intraoocyte injection of CTL/GDF-9 MO; c and d, GDF-9 expression (immunohistochemistry) in the CTL MO- (c) and GDF-9 MO-injected (d) follicles on d 4. Note that oocytes injected with GDF-9 MO exhibited significantly lower GDF-9 immunostain intensity compared with those with CTL MO. B, A representative image on d 4 is shown for each treatment group: a, CTL MO + CTL; b, CTL MO + GDF-9; c, GDF-9 MO + CTL; and d, GDF-9 MO + GDF-9, respectively. *Scale bar*, 100 μM. C and D, Effect of GDF-9 down-regulation on preantral follicle growth *in vitro*. CTL MO or GDF-9 MO was injected into the oocyte of the rat preantral follicles at d 0. At d 1, the follicles (diameter, 150–170 μm) were treated with or without GDF-9 (100 ng/ml) and FSH (10 ng/ml) and cultured for another 3 d (d 1–4). CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. The percentage change of follicular volume on day n of culture is defined as the volume difference between day n and d 0 (the day of microinjection) expressed as a percentage of the volume at d 0. Results represent the means ± SEM of a total of 20 follicles from four or five independent experiments. *Bars with different superscripts* are significantly different at $P <$ 0.05. Note that GDF-9 stimulated preantral follicle growth during a 4-d culture period. Although GDF-9 MO injection suppressed follicular growth, addition of GDF-9 to the culture media prevented the growth arrest induced by GDF-9 antisense on d 4.

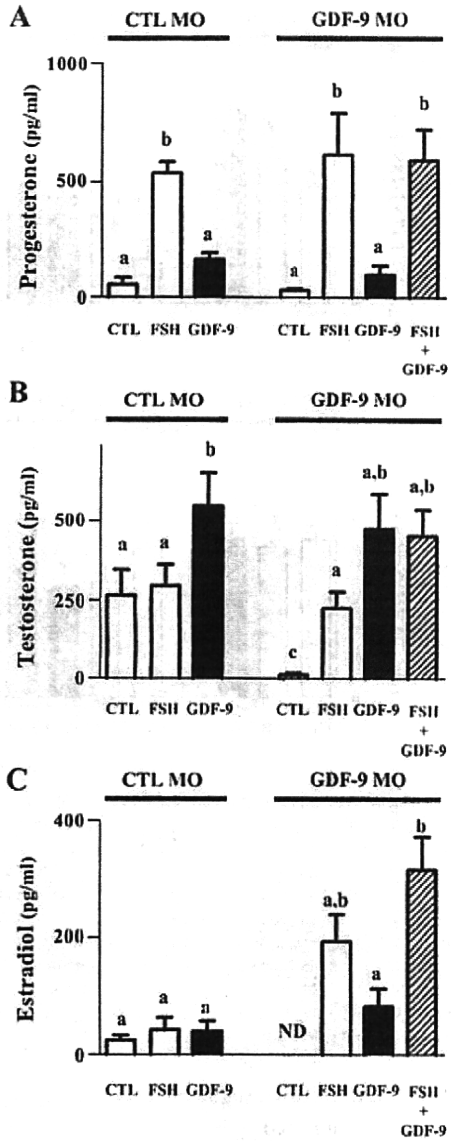

FIG. 3. Effect of intraoocyte injection of GDF-9 MO on steroidogenesis by preantral follicle *in vitro*. CTL/GDF-9 MO-injected preantral follicles at d 0 were treated with or without GDF-9 (100 ng/ml) and FSH (10 ng/ml) and cultured for another 3 d (d 1–4). CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. Concentrations of progesterone (A), testosterone (B), and estradiol (C) in the spent media on d 4 were measured. Data are the mean ± SEM of a total of 20 follicles from four or five independent experiments. ND, Not detected. *Bars with different superscripts* are significantly different at $P < 0.05$. Note that GDF-9 MO markedly decreased androgen biosynthesis, although this response was attenuated by exogenous GDF-9.

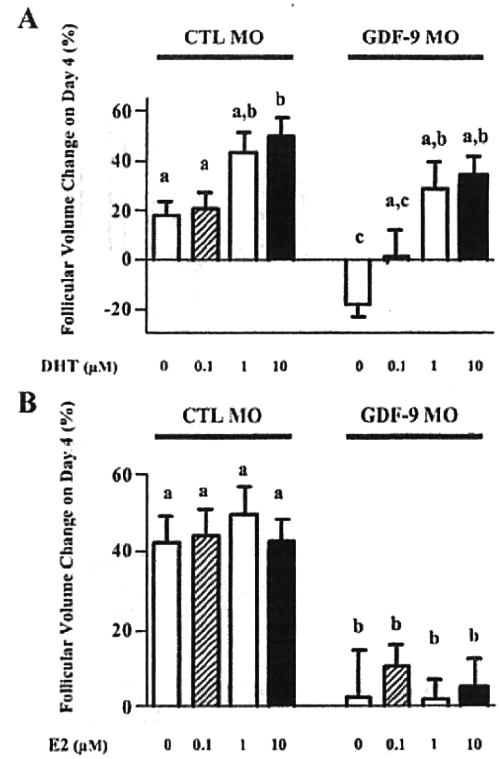

FIG. 4. Effect of DHT and estradiol on preantral follicle growth arrest induced by GDF-9 MO *in vitro*. CTL/GDF-9 MO-injected preantral follicles at d 0 were cultured with different concentrations of DHT (A; 0–10 μM), a nonaromatizable androgen, or estradiol (E2; B; 0–10 μM). The percentage change of follicular volume on d 4 of culture is defined as the volume difference between d 4 and d 0 (the day of microinjection) expressed as a percentage of the volume at d 0. Results represent the means ± SEM of a total of 20 follicles from four or five independent experiments. *Bars with different superscripts* are significantly different at $P < 0.05$. Note that the addition of DHT, not estradiol, to the culture media prevented the growth arrest induced by GDF-9 antisense on d 4.

MO-injected follicles ($P > 0.05$, CTL MO + FSH *vs.* CTL MO + CTL) but significantly suppressed GDF-9 MO-induced testosterone down-regulation ($P < 0.05$, GDF-9 MO + FSH *vs.* GDF-9 MO + CTL). GDF-9 appeared to be more effective in stimulating androgen production than FSH in the CTL MO-injected follicles [550.3 ± 108.5 pg/ml (CTL MO + GDF-9) *vs.* 293.3 ± 67.5 pg/ml (CTL MO + FSH), $P < 0.05$], although no significant difference was observed between the GDF-9 MO + GDF-9 and the GDF-9 MO + FSH group.

Although treatment with GDF-9 and FSH did not alter estradiol production in the follicles injected with CTL MO (Fig. 3C), GDF-9 MO markedly decreased estradiol production [< 10 pg/ml (GDF-9 MO + CTL) *vs.* 20.1 ± 7.3 pg/ml (CTL MO + CTL)]. In the GDF-9 MO-injected follicles, addition of GDF-9 into the culture media increased estradiol production [< 10 pg/ml (GDF-9 MO + CTL) *vs.* 84.2 ± 29.4 pg/ml (GDF-9 MO + GDF-9)]. FSH also augmented estradiol production in the GDF-9 MO-injected follicles irrespective of the presence of GDF-9 ($P < 0.01$).

Based on the results of Figs. 1 and 3, GDF-9 appeared to preferentially stimulate the production of testosterone rather than estradiol in the follicles during preantral to early antral transition.

Androgen action is involved in GDF-9-induced preantral follicle growth *in vitro*

To determine whether and how androgen modulates GDF-9-induced preantral follicles *in vitro*, the CTL MO- and GDF-9 MO-injected follicles were cultured with different concentrations of DHT (0–10 μM), a nonaromatizable androgen. DHT augmented the CTL-MO injected follicular growth in a concentration-dependent manner ($P < 0.05$; Fig. 4A). Ten micromoles of DHT was the most effective concentration in inducing a significant increase in preantral follicle growth in our culture sys-

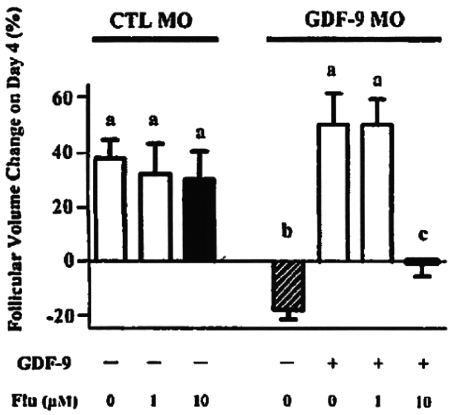

FIG. 5. Effect of AR antagonist on GDF-9-induced preantral follicle growth *in vitro*. CTL/GDF-9 MO-injected preantral follicles at d 0 were cultured with or without GDF-9 and different concentrations of flutamide (Flu; 1–10 μM), a specific AR antagonist. The results were expressed as change in follicular volume on d 4, as described in the legend of Fig. 4. Results represent the means ± SEM of a total of 20 follicles from four or five independent experiments. *Bars with different superscripts* are significantly different at $P < 0.05$. Note that, although exogenous GDF-9 prevented the follicular growth arrest by GDF-9 MO, addition of flutamide (Flu; 10 μM) to the culture media suppressed this response.

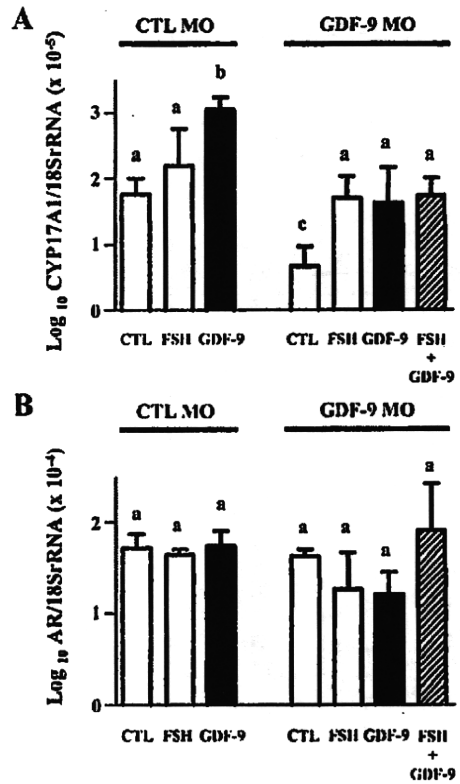

FIG. 6. Effect of GDF-9 down-regulation on CYP17A1 and AR expression *in vitro*. CTL/GDF-9 MO-injected preantral follicles at d 0 were treated with or without GDF-9 (100 ng/ml) and FSH (10 ng/ml) and cultured for another 3 d (d 1–4), and real-time quantitative PCR analysis of CYP17A1 (A) and AR (B) was performed. CTL, Preantral follicles cultured in the absence of GDF-9 and FSH. Total RNAs from three follicles (pooled from the same treatment group) were extracted, and the cDNAs were amplified as one sample. Results are represented as the logarithm of the means ± SEM of three to five independent experiments. *Bars with different superscripts* are significantly different at $P < 0.05$. Note that GDF-9 increased CYP17A1 mRNA levels in the preantral follicles, whereas GDF-9 down-regulation markedly decreased this response.

tem. In the GDF-9 MO-injected follicles, DHT also enhanced preantral follicle growth in a concentration-dependent manner (Fig. 4A). Although GDF-9 down-regulation suppressed follicular growth ($P < 0.01$), the addition of 1 and 10 μM of DHT to the culture media prevented this response ($P < 0.01$). In contrast, treatment with estradiol (0–10 μM) did not alter the growth of CTL-MO or GDF-9-MO injected follicles (Fig. 4B).

Furthermore, to examine the role of androgen action in GDF-9-induced preantral follicle growth, the CTL MO- and GDF-9 MO-injected follicles were cultured with or without GDF-9 and with different concentrations of flutamide (0–10 μM), a specific AR antagonist. Although exogenous GDF-9 prevented the follicular growth arrest caused by GDF-9 MO ($P < 0.01$), addition of flutamide (10 μM) to the culture media suppressed this response ($P < 0.01$; Fig. 5). Flutamide did not alter the growth of the CTL MO-injected follicles ($P > 0.05$). These results demonstrated that the effects of androgen on the GDF-9-induced preantral follicle growth were not due to aromatization to estradiol, and were inhibited by an antagonist to the androgen receptor.

GDF-9 is essential for the expression of follicular CYP17A1 mRNA *in vitro*

To determine by what means GDF-9 regulates follicular androgen action during this stage, real-time quantitative PCR analysis of CYP17A1 (mainly expressed in thecal cells) and AR (mainly expressed in granulosa cells) was performed on total RNAs from the MO-injected follicles. GDF-9, but not FSH, augmented CYP17A1 mRNA levels in the follicles injected with CTL-MO ($P < 0.05$, CTL MO + GDF-9 *vs.* CTL MO + CTL), whereas GDF-9 MO markedly decreased this response ($P < 0.05$, GDF-9 MO + CTL *vs.* CTL MO + CTL; Fig. 6A). The down-regulation of CYP17A1 transcript by GDF-9 MO was prevented by exogenous GDF-9 ($P < 0.05$, GDF-9 MO + GDF-9 *vs.* GDF-9 MO + CTL), and CYP17A1 mRNA abundance in GDF-9 MO + GDF-9 was not different from those in CTL MO + CTL ($P > 0.05$). FSH also augmented CYP17A1 mRNA levels in the GDF-9 MO-injected follicles ($P < 0.05$, GDF-9 MO + FSH *vs.* GDF-9 MO + CTL). Addition of GDF-9 or GDF-9 down-regulation did not affect AR mRNA expression in the preantral follicles (Fig. 6B). No significant difference in AR mRNA abundance was observed between the CTL MO + CTL, CTL MO + GDF-9, GDF-9 MO + CTL, and GDF-9 MO + GDF-9 group ($P > 0.05$).

Discussion

Although oocyte-somatic cell communication is important in early follicular development, our knowledge of the precise role of the oocyte-derived factor GDF-9 during the preantral-early antral transition is incomplete. In the present study, we have shown for the first time that: 1) intraoocyte injection of GDF-9 MO antisense suppressed rat preantral follicle growth *in vitro*, whereas GDF-9 enhanced follicular development; 2) GDF-9 augmented testosterone production in preantral follicles; 3) GDF-9 MO suppressed androgen production and CYP17A1 mRNA expression in cultured follicles, a response attenuated by exogenous