色体異常が誘導されると報告されている[20, 21]。操作者による差も大きく、今後改良がもっとも必要な事項であると考えられる。

① 機 械 的

機械的、いわゆる mechanical による継代方法[1]が、染色体変異が少なく、分化能や未分化性の維持がもっとも良いと、ヒト ES 細胞研究者の間では信じられている。顕微鏡下で、形のよいコロニーを探し、まず、その周りのフィーダーを先の細いプラスチックパスツールで除き、コロニーを50～100程度の細胞集団になるようにプラスチックパスツールで小さく刻んでからピックアップし、新しい MEF の上に播種する。この手法は熟練と時間を要し、また、大容量での培養は難しい[3]。最近、インビトロジェンから専用のスクレーパー（StemPro EZPassage）が発売された。時間と熟練は要さずに継代できるがコストは高くなる。

② コラゲナーゼ

0.1％コラゲナーゼ IV[13] とセルスクレーパーを用いる方法が一般的に使用されている。培地を除き、0.1％コラゲナーゼ IV を 1 ml 加えて37℃のインキュベーターに入れて約10分程度処理する。時間は酵素活性によるが、時々、顕微鏡下で観察し、フィーダーとコロニーの間に間隙ができる程度になったら、コラゲナーゼを吸引し、新しい培地を2～3 ml 加えてからセルスクレーパーで細胞をはがす。培地をさらに 7 ml 程度加えてフィーダーとコロニーを引き離すように、ピペッティングを数回行う。シェフィールド大学では、コラゲナーゼとガラスビーズを使用している。

③ EDTA

0.1～0.2％ EDTA で処理すると、適度な大きさにコロニーが分散され、未分化なコロニーだけが回収できる。分化した細胞集団から未分化なコロニーを選択する際に使用できる。しかし、EDTA に対する感受性は細胞株によって異なり、シングルセルになる細胞株もあり、すべての細胞株に使用できるわけではない。

④ トリプシン／EDTA

ヒト ES 細胞はトリプシンに弱く、0.05％ trypsin /1 mM EDTA を使用する。通常の継代に使用されている細胞株もあるが、FACS による解析の際にシングルセルにするために使用されることが多い。

⑤ トリプシン／コラゲナーゼ（CTK）

日本国内では末盛ら[3] により開発された［Ca^{2+}］で活性を弱めたトリプシンとコラゲナーゼとの混合液 CTK が使用されていることが多い。処理時間も短く簡便であるが、均一で適度な大きさのコロニーにするためには若干の熟練を要する。

⑥ ディスパーゼ

合同酒精が自然界より分離選択したバチルス属の菌株の培養液より精製したプロテアーゼで、トリプシンやコラゲナーゼより穏やかに細胞を分散できる。Thomson らによる樹立時には、機械的方法かディスパーゼが用いられている。当セルバンクにおいては、現在、iPS 細胞の継代にはディスパーゼを使用している。

⑦ アキュターゼ™

ミリポアから販売されているプロテアーゼ、コラーゲン分解酵素による細胞剥離剤とされているが組成は明らかにされていない。ヒト ES 細胞は通常シングルセルとなると増殖できないが、この分散液を使用した場合には、シングルセルにしても細胞が増殖できる。クローナルアッセイなどの場合に使用されている。

⑧　ROCK インヒビター

　細胞分散液ではないが、シングルセルにした際のアポトーシスを阻害する薬剤として、笹井ら[22]により報告された。遺伝子導入後のクローニングなど、シングルセルにする必要がある際に使用されている。

4. 未分化性の同定法について

　未分化なヒト ES 細胞に発現するマーカー分子や表面抗原などが報告されているが、未分化性を同定する決定的なマーカー分子は見つかってない。現状では、形態からの判断がもっとも確実であると言われている。

① 形　　態

　細胞質がほとんどなく、丸い核を持った細胞がコンパクトに集合したコロニーを形成し、細胞間境が明瞭でない特徴的な様相を呈する[17]（図 1）。

② アルカリフォスファターゼ活性の同定

　未分化なマウス、サル、ヒトの ES 細胞は、組織非特異的アルカリフォスファターゼ活性を示すことが知られている。その機能については、明らかとなっていない。

③ FACS による細胞表面抗原の同定

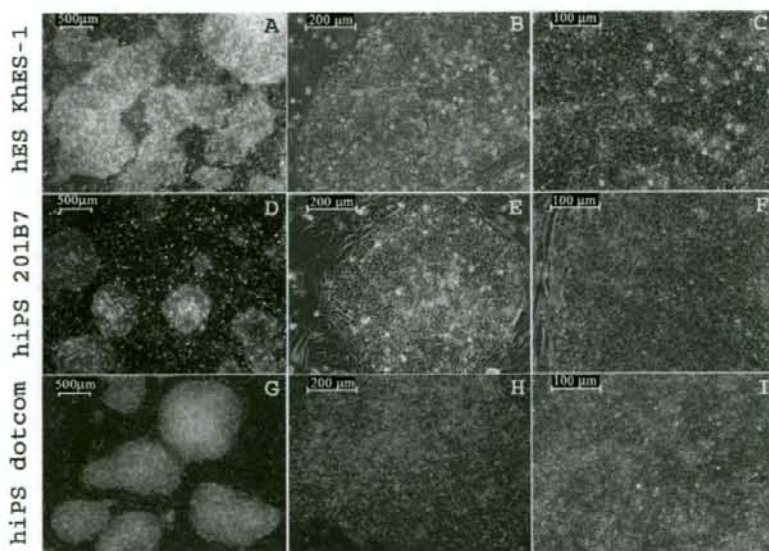　ヒト Embryonal carcinoma（EC）に発現する糖脂質である stage specific embryonic antigen（SSEA）-3、

図 1：
A～C：hES khES-1 京都大学再生医科学研究所 により分配されたヒト ES 細胞 khES-1。
D～F：hiPS 201B7；物質－細胞統合システム拠点 iPS 細胞研究センター／再生医科学研究所・山中伸弥教授から共同研究契約により提供されたヒト iPS 細胞　201B7 clone。
G～I：hiPS Dotcom; 国立成育医療センター生殖医療研究部梅澤明弘部長より寄託されたヒト iPS 細胞 dotcom clone。

SSEA-4、糖タンパクである Tra-1-60、Tra-1-81 が広く使用されている[23]。SSEA-1 は、マウスにおいては未分化な ES 細胞において発現するが、ヒトにおいては、分化した ES 細胞において発現する。SSEA-3、SSEA-4 は、未分化なヒト ES 細胞にも発現するが、赤血球にも発現する。一般的には、SSEA-1 と、SSEA-3 あるいは SSEA-4 が調べられている。しかし、SSEA-3 が発現しているからといって SSEA-4 が発現しているとは限らないため、これらの抗体をセットで解析することが望ましい。

④ RT-PCR による遺伝子発現の解析

現在、多くの転写因子が未分化性維持に機能していることが報告されている。なかでも、OCT3/4、NANOG、SOX2 はマウスならびにヒト ES 細胞の未分化マーカー遺伝子として認識されている[24]。そのほかに、REX-1、TGDF、FGF-4、LIN28、LEFTB なども未分化なヒト ES 細胞に発現することが報告されている。ヒト ES 細胞研究 Initiative project による世界で樹立された59株のヒト ES 細胞における遺伝子発現解析の結果が報告されているので[12]、参照されたい。

5. 培養の実際

ヒトES、iPS 細胞は、継代ごとに、あるいは、操作者により細胞の状態が異なってしまうことはよく見られ、大変扱いが難しい。操作者の腕が問われる細胞である。油断をすると、ほとんどの細胞が分化してしまう。実験をする上でも、毎回同じ結果を得ることが難しいため、指導する研究者は間違いが起きないよう十分に監督する必要があるだろう。株によって相当に特徴が異なるため、あまり扱いにくいようであれば、株を変えることをお勧めする。以上、簡単ではあるが、ヒト ES、iPS 細胞の培養方法についての概要をまとめてみた。本総説が、多くの研究者によるより良い成果産出

の一助となることを願う。

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Standardization of human embryonic stem (ES) cell and induced pulripoent stem (iPS) cell research in Japan

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Abstract   In 1998, human embryonic stem (ES) cells have been established.   Since then, the human ES cells have been used as a tool for understanding the mechanisms in human development and regeneration application research in the world. However, in Japan, not so many searchers have used human ES cells.   It is caused from several issues.   In 2007, human induced pluripotent stem (iPS) cells have been developed.   The situation in Japanese stem cell research have been changing.   As the characteristics of Human iPS cells are quite similar to those of human ES cells, researcher can use the protocols of human ES cells for iPS cell research.   In this review, I have summarized the culture and characteristics of human ES and iPS cells.

Key Words: human ES cells, human iPS cells, feeder cells

マウス ES 細胞の無血清培養法

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【要約】　　　マウス胚性幹（ES）細胞は、哺乳類の発生過程における培養モデルとして、分子生物学的、生化学的解析に用いられている。しかし、培養条件下における未分化状態の維持、及び分化制御はいまだ不十分である。我々の研究グループは、未分化マウス胚性幹細胞の既知の成分よりなる無血清培地の確立を行った。従来の血清入りの条件とこの無血清培養条件を比較したところ、白血病阻害因子（LIF）の効果が異なっており、この無血清条件下では LIF は未分化性の維持のみならず細胞の生存、増殖に働いていることが示された。また、細胞外マトリックスの効果を検証したところ、コラーゲンが未分化性の維持に適していることがわかった。このように、無血清培養法はマウス ES 細胞における生理活性物質の影響を解析するのに適しており、今後、その応用による分化誘導法の開発が期待できる。ここで、その方法について概説する。

キーワード：　ES 細胞、無血清培養、細胞外マトリックス

序　　文

胚性幹（ES）細胞[1,2]は、成体を構成する全種類の細胞種に寄与することができ、*in vitro* においても同様の分化能を示すと考えられている。このため、ES 細胞は哺乳類・発生過程の解析ツールとして、分子生物学的、生化学的解析に用いられて

いる[3,4]。ES 細胞を *in vitro* における生物学的解析のための確実な培養モデルとして使用するためには、未分化状態の維持、及び分化制御が重要であると考えられる。しかし、未だその制御技術は不十分である。その一因として、現在、一般的な ES 細胞の培養条件においては支持細胞や動物血清が用いられていることが挙げられる。これら動物由来の不特定因子が存在している培養条件では、安定した再現性が得られず、添加した生理活性物質の影響を正確に解析することは困難である。ES 細胞の未分化性ならびに分化を制御するために、単純かつ既知の因子よりなる培養液の開発、また、そのような条件下における生理活性物質の詳細な

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解析が求められている。

　我々は、これまで種々の無血清培地の開発、ならびにそれらを用いた各種細胞の培養法の確立を行ってきた[5,6]。今回、支持細胞を用いず未分化性を長期に維持できるマウスES細胞の無血清培養条件を確立した。また、その過程で白血病阻害因子（LIF）と細胞外マトリックス（ECM）の効果を検証した[7]ので、これを概説する。

1. 未分化マウスES細胞の無血清培養条件の探索

　一般的なマウス ES 細胞の培養は、まずマウス胎児由来線維芽細胞を播種し、翌日に、それを支持細胞としそこに播種する。血清を含む培地で2～3日ごとに継代培養することにより未分化状態を維持できる[1,2]。支持細胞と血清を培養液から除去することは困難であると考えられていたが、支持細胞から分泌される LIF が未分化性維持に重要であることが明らかとなり、支持細胞の代わりにLIF を用いることが可能となった[8]。1999年に行われた Wiles らは、無血清培養条件で3代以上培養すると分化した細胞が出現してしまうと報告している[9]。この事から、血清には ES 細胞の未分化状態を維持するためのさらに重要な因子が含まれていると考えられていた。2003年に、Smith らのグループが神経細胞培養用のサプリメントである N2ならびに B27 と、BMP4 ならびに LIF を用いることで、支持細胞だけでなく血清も使用することなくES細胞の自己複製能を維持したまま培養できるという結果を報告し[10]、ES 細胞が血清を用いずに未分化性を維持できることが明らかとなった。

　一方、我々も独自に未分化 ES 細胞の無血清培養に成功した。マウス ES 細胞用に ESF 基礎培地を開発し、その培地に 7F（亜セレン酸ナトリウム、メルカプトエタノール、エタノールアミン、インシュリン、トランスフェリン、オレイン酸—BSA、

LIF）を加えた ESF7 培地をマウス ES 細胞の未分化維持用の培地として採用した。血清入りの培地から ESF7 培地に移して、ES 細胞の D3 株（ES-D3細胞）を3代ほど培養すると、ほぼ全てのコロニーが、境界に突起のない、細胞間隙が不明瞭なコロニーとなった（培養6日目、図1A）。これは、未分化 ES 細胞に特有の様相である。一方、一般に使用されている血清入りの ES 細胞培養用培地 CEM で2～3日は未分化状態を維持できるが、6日間培養したものでは一部の細胞が分化してコロニーより出て成長し、線維芽様、上皮様、あるいは神経様の細胞が観察される（図1B）。ESF7 培地中で一ヶ月間培養した細胞において未分化マーカーの検出を行ったところ、アルカリホスファ
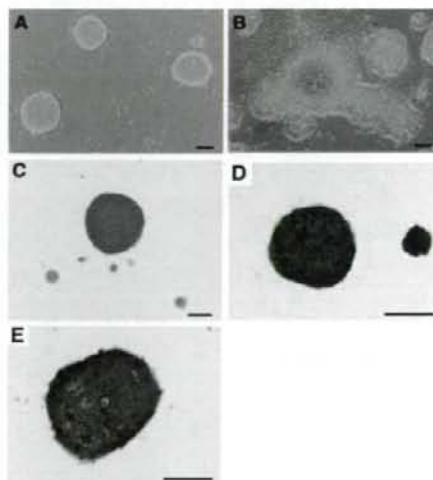

図1　無血清培養条件での ES-D3 細胞の未分化性.
(A): ESF7 培地中での ES-D3 細胞の位相差顕微鏡像. (B): CEM培地中での ES-D3 細胞の位相差顕微鏡像. (C): ESF7 培地中での ES-D3 細胞におけるアルカリホスファターゼ活性染色像. (D): ESF7 培地中での ES-D3 細胞におけるOct3/4 の免疫染色像. (E): ESF7 培地中でのES-D3 細胞における SSEA-1 の免疫染色像. Bars; 100 μm (In Vitro Cell Dev Biol Anim. 41, 19-28, 2005. Figure1 A, B, C, E, G より)
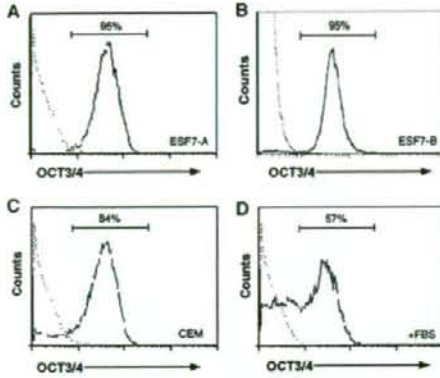
図2 Oct3/4 タンパク質のフローサイトメトリーによる発現プロファイル.
(A): ESF7 培地にて 6 ヶ月間培養した ES-D3 細胞の発現プロファイル. (B): ESF7 培地にて2年間培養した ES-D3 細胞の発現プロファイル. (C): CEM 培地にて 6 日間培養した ES-D3 細胞の発現プロファイル. (D): ESF 基礎培地に FBS と、2-メルカプトエタノールを添加して 6 日間培養した ES-D3 細胞の発現プロファイル. (In Vitro Cell Dev Biol Anim. 41, 19-28, 2005. Figure 4より)

ターゼ活性はほとんどの細胞が陽性（図 1C）であり、転写因子である Oct3/4（図 1D）、細胞表面抗原であるSSEA1（図 1E）も同様にほとんどの細胞で陽性であった。さらに Oct3/4 の発現を flow cytometry によって解析すると、ESF7 培地で 6 ヶ月以上培養したもの（図 2A）、2 年以上培養したもの（図 2B）、ともに95%以上の細胞が陽性であった。一方、CEM 培地では84%の陽性率であり、（図 2C）、LIF をのぞくと、57%まで陽性率は低下する（図 2D）。以上の結果から、ESF7 培地を用いて ES-D3 細胞の未分化性を効率的に維持できることが示された。また、株化されていない 129/SV マウス由来の ES 細胞（継代数10）を ESF7 培地へ移して培養を行うと、ES-D3 細胞同様に未分化様コロニーが観察され（図 3A）、これらのコロニーは、ES 細胞の未分化マーカーの一つであるアルカリホスファターゼ活性が（図 3B）、Oct-3/4 であった（図 3C）。また、この株化していない ES 細胞の ESF7 培地での増殖能を検討すると（図 3D）、
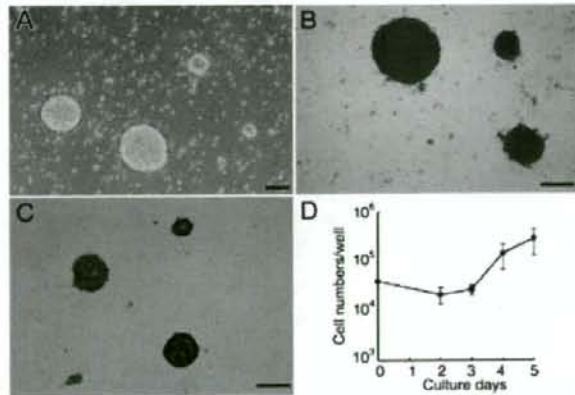

図3 株化されていない 129Sv マウス由来の ES 細胞の無血清培養条件下の未分化性.
(A): ESF7 培地で 3 日間培養した 129Sv ES 細胞（継代数12）の位相差顕微鏡像. (B): ESF7 培地中での 129Sv ES 細胞におけるアルカリホスファターゼ活性染色像. (C): ESF7 培地中での 129Sv ES 細胞における Oct3/4 の免疫染色像. (D): 129Sv ES 細胞の増殖曲線. 129Sv ES細胞を 4×10⁴ cells/well（24 well plate）にて播種し、細胞数を 1 日毎に計測. 値は平均値±標準偏差 Bars; 100 μm. (In Vitro Cell Dev Biol Anim. 41, 19-28, 2005. Figure 6 より)

倍加時間は14時間であり、十分な増殖が得られることがわかった。以上の結果は、種々のES細胞の未分化性の維持において、ESF7培地が有効であることを示している。

2. 無血清培養条件下におけるES細胞に対するLIFの効果

インターロイキン6ファミリーに属するサイトカインであるLIFはgp130と、低結合能のレセプターからなるヘテロ受容体と結合し、細胞内のシグナルを活性化させる。これまでLIFは、マウスES細胞の未分化性の維持に対して重要であることが示されている[8]。その下流シグナル経路であるSTAT3経路の活性化は特に、マウスES細胞の未分化性の維持に対して必須であることが示されている[12, 13]。一方、別の下流シグナルであるMAPK経路の活性化は、ES細胞の未分化性に、抑制的に働くことが報告されている[14]。また、別の下流シグナルであるPI3K経路[15]やSrcファミリーの活
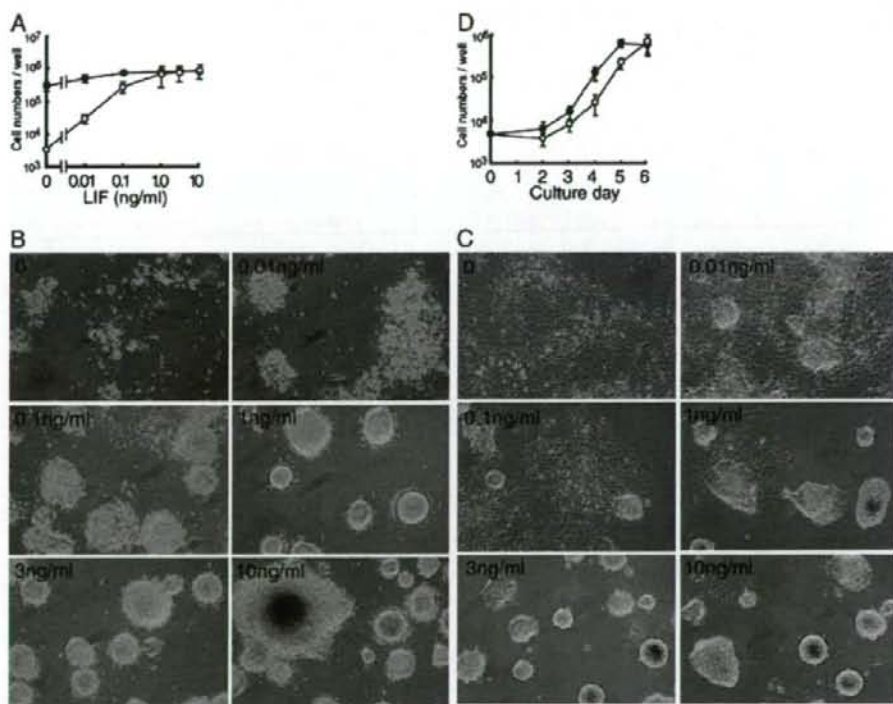

図4　無血清条件下のES細胞におけるLIFの効果.
　　　(A): ESF7培地中（白丸）、CEM培地中（黒丸）における各LIF濃度で添加した条件にて、ES-D3細胞を5×10³ cells/well（24well plate）にて播種し、細胞数を5日目に計測. 値は3回の計測の平均値±標準偏差. (B): 各LIF濃度のESF7培地で3日間培養したES-D3細胞の位相差顕微鏡像. (C): 各LIF濃度のCEM培地で3日間培養したES-D3細胞の位相差顕微鏡像. (D): ES-D3細胞の増殖曲線. ES-D3細胞を5×10³ cells/well（24 well plate）にて播種し、細胞数を1日毎にtriplicateさせて計測. 値は平均値±標準偏差 Bars; 100 μm. (In Vitro Cell Dev Biol Anim. 41, 19–28, 2005. Figure 2 より)

性化[16] も未分化性の維持に促進的に働くことが報告されている。LIF によるマウス ES 細胞の未分化性の維持は、これらの下流シグナルの活性化のバランスで成り立っていると考えられている。

　無血清培養条件 ESF7 において LIF の効果を検討したところ、これまで知られていなかったマウス ES 細胞に対する生存、増殖に対する機能が明らかとなった。血清を含む培養条件 CEM、あるいは ESF7 培地で LIF の濃度の ES-D3 細胞の増殖能に及ぼす影響を調べた。その結果、ESF7 では培養 5 日後での ES-D3 細胞の細胞数は LIF の濃度依存的に増大した。一方、CEM では LIF の濃度を変化させてもほとんど細胞数に変化はなかった（図 4A）。細胞の形態は、ESF7 では、LIF の非存在下あるいは低濃度では、ほとんどが細胞死を示す縮小した細胞形態が観察されたが、高濃度の LIF 存在下では細胞死はほとんど見られず、未分化の形態を維持していた（図 4B）。CEM では、LIF の非存在下あるいは低濃度では、ほとんどのコロニーにおいて分化した細胞形態が観察されたが、高濃度の LIF 存在下では、未分化の形態を呈していた（図 4C）。細胞増殖は、ESF7 と CEM でその倍加時間はほぼ同等であった（図 4D）。以上の結果から、無血清培養条件下において解析することにより、LIF は ES 細胞の未分化性の維持と増殖性の両方に関与していることが明らかとなった。さらに、ESF7 で、LIF 存在下、非存在下の条件で培養した細胞の DNA を Propidium Iodide で染色して flow cytometry で解析すると、LIF 存在下では、G0/G1 期を示す DNA 量以下にはピークはほとんど検出されなかったが、LIF 非存在下では、G0/G1 期を示す DNA 量以下のピークが多くなり、S 期や G2/M 期を示す DNA 量のピークは減少していた（図 5）。以上の結果は、ESF7 から LIF をのぞくと、DNA の断片化によるアポトーシスを起こすことを示しており、LIF は ES 細胞の生存に必要な役割を持っていることを示している。また、血清中には未分
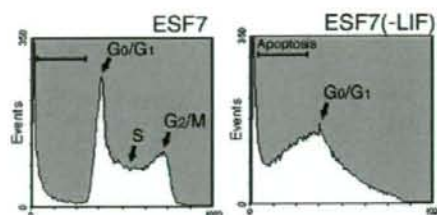

図 5　フローサイトメトリーによる ES-D3 細胞における DNA 量の解析.
ES-D3 細胞を LIF あり（左パネル）／なし（右パネル）の ESF7 培地にて 2 日間培養. その細胞の DNA を PI にて染色、フローサイトメトリーにて解析. 矢印はそれぞれ G1 期、S 期、G2 期の細胞の DNA 量のピークを示す.（In Vitro Cell Dev Biol Anim. 41, 19-28, 2005. Figure 3より）

化性の維持には働かないが、ES 細胞の増殖を促進する因子、アポトーシスを抑制し、分化を促進する因子が含まれていることが示唆される。

　今後は、これら血清含有条件/無血清条件で異なったマウス ES 細胞の LIF の下流シグナルの挙動を調べることで、これらのシグナルの関与がより詳細に明らかになることが期待できる。

3. 無血清条件下における ECM の ES 細胞に対する効果

　細胞外マトリックス（ECM）成分は、移動や増殖など細胞の挙動に、多大な影響を与えることが知られている[17, 18, 19, 20]。一部の種類の幹細胞においては、ECM が自己複製能に対して重要な機能を果たしていることがこれまでに示されている[21]。また、ラミニンやフィブロネクチンなど比較的発生初期に発現がみられる ECM 成分、またはその主要なレセプターであるインテグリンを欠損した ES 細胞では、分化異常が見られる[22, 23] ことから、細胞分化に重要な役割を持っていることが推測される。

　筆者らは ES 細胞の無血清条件培養法を確立する
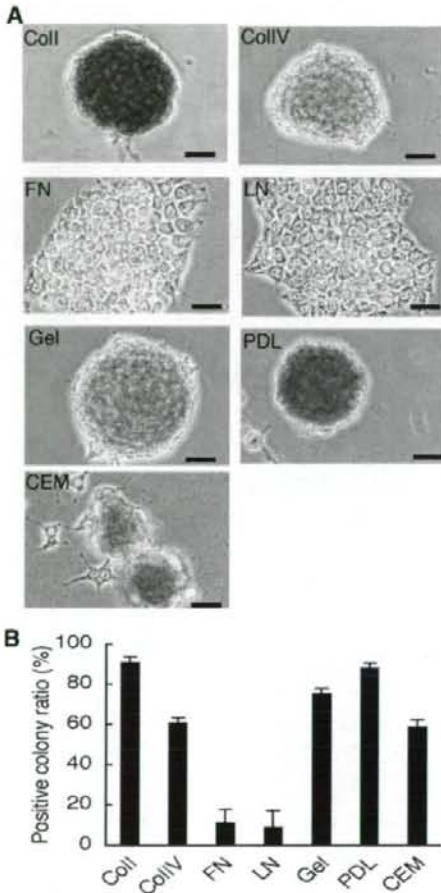
にあたり、ES 細胞の未分化維持に適する ECM 成分の探索を行った。さらに、それら ECM 成分の

図 6　各 ECM 上での ES-D3 細胞の未分化性.
(A): 各細胞外マトリックス上、ESF7 培地にて 4 日間培養した ES-D3 細胞のアルカリホスファターゼ活性染色像. Bars, 50 μm. (B): 各細胞外マトリックス上、ESF7 培地にて 4 日間培養した ES-D3 細胞のアルカリホスファターゼ活性染色陽性コロニー数の割合. 割合の値は100以上のコロニーから計測し、平均値±標準誤差を示す (N = 5). (Stem Cells. 25, 3005–3015, 2007. Figure 1 A, B より)
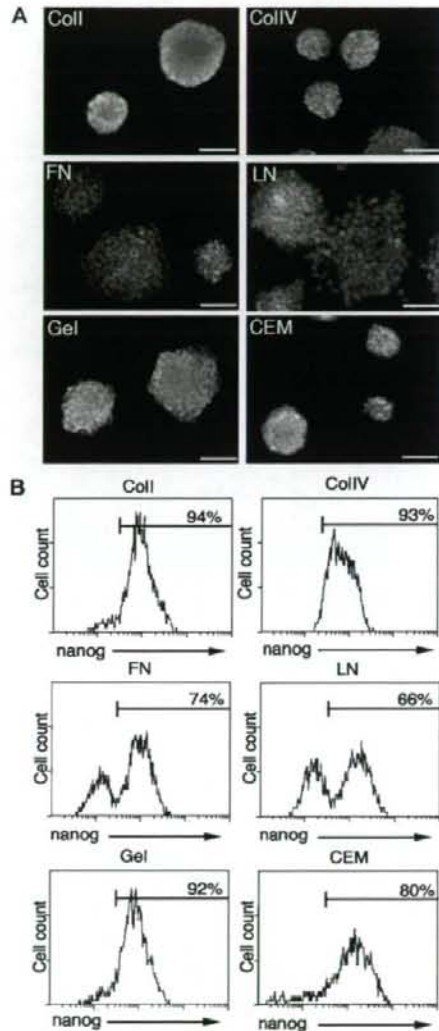

図 7　各 ECM 上での ES-D3 細胞の Nanog タンパク質の発現. (A): (A): 各細胞外マトリックス上、ESF7 培地にて 4 日間培養した ES-D3 細胞の Nanog タンパク質の免疫染色像. Bars, 50 μm. (B): 各細胞外マトリックス上、ESF7 培地にて 4 日間培養した ES-D3 細胞の Nanog タンパク質のフローサイトメトリーによる発現プロファイル. (Stem Cells. 25, 3005–3015, 2007. Figure 1 C, D より)

ES 細胞に対する影響も解析した[24]。ESF7 培地を用いて、各種細胞外マトリックス成分（I 型コラーゲン、IV 型コラーゲン、フィブロネクチン、ラミニン、ゼラチン）でコートしたディッシュ上で ES-D3 細胞を培養し、未分化マーカーの検出を行った。ES 細胞の未分化マーカーの一つであるアルカリフォスファターゼ（AP）活性を基質染色により検出した。AP 活性は、I 型コラーゲン、IV 型コラーゲン、ゼラチンにおいては高い陽性率を示したが、ラミニンやフィブロネクチン上では、陽性率は低下した（図 6A, B）。未分化マーカーである Nanog タンパク質の抗体を用いて免疫染色法、Flow Cytometry 法によって、その発現を同定すると、フィブロネクチンやラミニン上において一部の細胞においてその発現が低下していた（図 7A, B）。以上の結果から、I 型コラーゲン、IV 型コラーゲン、ゼラチン上では ES 細胞は未分化に保たれ、フィブロネクチン、ラミニン上では分化が促進されていることが示された。この結果を踏まえ、ES 細胞の未分化性を維持した培養は各種コラーゲン上で行うことが適切であると考えた。

お わ り に

無血清培地 ESF7 を用いて、支持細胞を用いることなく、マウス ES 細胞株の未分化性を維持でき、また、長期間に渡って継代可能である。この培養法は、マウス ES 細胞に対する外因性の生理活性物質の影響を調べるのに適しており、これまでの結果では、LIF が ES 細胞の未分化性の維持に関わるのみならず、生存、増殖にまで関わる必須の分子であることが示されている。さらに、ECM 成分では、コラーゲンでは未分化性を維持できるが、フィブロネクチンやラミニンは LIF 存在下であっても、ES 細胞の分化を促進することがわかった。また、今回は示していないが、培地の組成、使用する ECM を変化させることで、マウス ES 細胞を各種

の細胞系列へと特異的に分化誘導することも可能である[11,25]。ES 細胞を不特定の因子を含まない条件で培養することは、ES 細胞の効率的な未分化維持、分化制御を可能とし、これらの分子的機構を解明するのに有効であると考えられる。

近年、ヒト ES 細胞や iPS 細胞の再生移植医療への応用が考えられている。しかし、支持細胞や動物血清を用いる従来の培養方法では、ウイルスやヒトの免疫系に対して抗原となりうる物質を含んでおり[26]、移植医療に使用することは安全面に問題がある。そこで、これらの動物由来因子を含まない培養条件の開発が必要となっている。今回紹介したマウスにおける無血清培養法を改良して、古江らは、ヒト ES 細胞用の無血清培地 hESF9 を開発した[27]。この培養法は、添加因子の正確な影響を高感度に解析でき、効率的な分化培養を行うのに利点があるとともに、ウイルスなどの病原体や特定の抗原物質を含まない再生移植医療に適していると考えられ、今後期待される。

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マウス ES 細胞の無血清培養法

Chemically defined serum-free culture for mouse embryonic stem cells

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Abstract   Mouse embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocysts, which differentiate into all three germ layers of the developing mouse embryo. Experimental manipulation of mouse embryonic stem cells *in vitro* and *in vivo* provides a model of mammalian development that is amenable to molecular and cellular analysis. We have developed a chemically defined serum-free medium which can maintain mouse embryonic stem cells in undifferentiated phenotype for more than two years. Under the serum-free culture conditions, leukemia inhibitory factor is sufficient to stimulate cell proliferation, inhibited cell differentiation, and maintained self-renewal of ES cells although the proliferation effect of LIF was not observed in the presence of serum. This serum-free culture method may be useful to study the biological effect of various factors on mouse ES cells. We have introduced this culture method here.

The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice

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Membrane fusion is an essential step in the encounter of two nuclei from sex cells—sperm and egg—in fertilization. However, aside from the involvement of two molecules, CD9 and Izumo, the mechanism of fusion remains unclear. Here, we show that sperm–egg fusion is mediated by vesicles containing CD9 that are released from the egg and interact with sperm. We demonstrate that the CD9$^{-/-}$ eggs, which have a defective sperm-fusing ability, have impaired release of CD9-containing vesicles. We investigate the fusion-facilitating activity of CD9-containing vesicles by examining the fusion of sperm to CD9$^{-/-}$ eggs with the aid of exogenous CD9-containing vesicles. Moreover, we show, by examining the fusion of sperm to CD9$^{-/-}$ eggs, that hamster eggs have a similar fusing ability as mouse eggs. The CD9-containing vesicle release from unfertilized eggs provides insight into the mechanism required for fusion with sperm.

fertilization | membrane fusion | EGFP | exosome

Fertilization is an essential process that naturally produces a cell capable of developing into a new individual. It consists of sequential events, including membrane fusion of sperm and egg (1). Despite the importance of understanding fertilization in controlling human reproduction and preserving endangered species, the molecular basis underlying the fusion remains a mystery, however. Previously, we reported that a tetraspan-membrane protein (tetraspanin), CD9, is expressed on the egg plasma membrane and is required for sperm–egg fusion (2–4). A role of CD9 in other fusion events also has been demonstrated (5). When sperm are added to eggs from CD9$^{-/-}$ females, the sperm bind to the egg plasma membrane normally, but fusion is severely impaired (2–4). Two recent observations suggest that CD9 plays a role in the organization of egg membrane. First, CD9 is transferred from the egg to the fertilizing sperm present in the perivitelline space (PVS) (6), suggesting the involvement of a process similar to trogocytosis, a mechanism of cell-to-cell contact-dependent transfer of membrane fragments (7). Second, CD9 deficiency alters the length and density of microvilli on the egg plasma membrane (8). CD9 is also known to be a component of exosomes, membrane vesicles released from a wide range of cells (9, 10). Despite its relationship to CD9, the involvement of exosome release in sperm–egg fusion remains unknown. In the present study, we analyzed the potential of enhanced green fluorescent protein (EGFP)-tagged CD9 (CD9-EGFP) as a reporter protein to study sperm–egg fusion in living mouse eggs.

Results

To observe the movement of CD9 during sperm–egg fusion, we generated a transgenic mouse line that expressed CD9-EGFP only in eggs (Fig. 1A), and converted to the genetic background of CD9$^{-/-}$ mice by mating mice. Western blot analysis using anti-CD9 monoclonal antibody (mAb) revealed that an expected CD9-EGFP with a molecular mass of 51 kDa (CD9 and EGFP
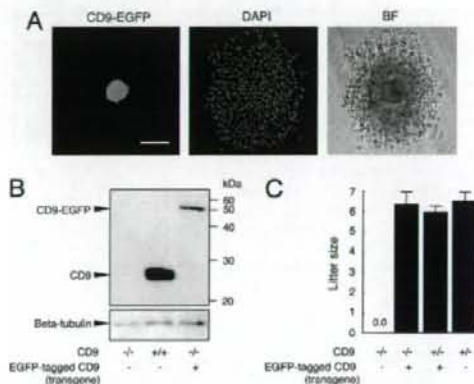

Fig. 1. Generation of mice expressing CD9-EGFP in eggs. (A) CD9-EGFP specifically expressed in eggs with mouse ZP3-promoter. Cumulus oocyte complex from Tg$^+$CD9$^{+/+}$ oviducts was collected at 14 h after injection of human chorionic gonadotropin. Nuclei of an egg and cumulus cells were counterstained with DAPI. (Left) CD9-EGFP. (Center) DAPI. (Right) Bright field. Scale bar: 100 μm. (B) Western blot analysis for eggs collected from CD9$^{-/-}$, CD9$^{+/+}$, and Tg$^+$CD9$^{-/-}$ mice. The same amounts, including 30 eggs of each lysate, were examined by anti-CD9 and anti-beta-tubulin mAbs (internal control). (C) Litter sizes of CD9$^{-/-}$ (n = 31), Tg$^+$CD9$^{-/-}$ (n = 35), Tg$^+$CD9$^{+/-}$ (n = 16), and CD9$^{+/-}$ mice (n = 15) (mean ± SEM). The numbers of females examined are in parentheses.

contributing to 24 and 27 kDa, respectively) was expressed in the eggs collected from Tg$^+$CD9$^{-/-}$ mice; however, the amount of CD9-EGFP expressed in CD9$^{-/-}$ eggs was estimated to be 10% of that of endogenous CD9 in the CD9$^{+/+}$ eggs (Fig. 1B). Despite the small amount of CD9-EGFP expressed in eggs, CD9-EGFP demonstrated the ability to reverse the sterility of CD9$^{-/-}$ females (Fig. 1C). The numbers of pups obtained from Tg$^+$CD9$^{-/-}$ females (6.4 ± 0.5) were similar to those from

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Fig. 2. Identification of secretory vesicles containing CD9 from unfertilized eggs. *A*, A single confocal image showing CD9-EGFP in unfertilized Tg⁺CD9⁻/⁻ eggs (E), including the PVS (*), zona pellucida (Z), and the outer margin of the zona pellucida (dotted line). (*Left*) Bright field. (*Right*) CD9-EGFP. *Lower* are enlarged images of the boxed areas. (*B*) Western blot analysis for eggs mechanically fractionated as shown in the diagram: zona-intact CD9⁻/⁻ eggs (E) (10 eggs per lane) and zona-free CD9⁺/⁺ eggs (10 eggs per lane). The medium (R) containing the remnant material from 40 eggs treated with a piezo manipulator was loaded in each lane. (*C*) Immunoelectron-microscopic analysis of CD9⁺/⁺ eggs. The zona-intact CD9⁺/⁺ eggs were examined using anti-CD9-mAb and 5-nm gold beads conjugated with anti-rat IgG Ab. Left panel: Image including CD9-containing vesicles (*), microvilli (arrowheads), zona pellucida (Z), perivitelline space (PVS), and egg (E). (*Right*) An enlarged image of the boxed region in the left panel. Scale bar: 200 nm. (*D*) RT-PCR for CD9, GM3 synthase, and glyceraldehyde-3-phosphate dehydrogenase transcripts in CD9⁺/⁺, CD9⁻/⁻, and

Tg⁺CD9⁻/⁻ eggs. The same amounts, including 50 eggs in each reaction, were examined. The right end lanes are negative controls in which RT was removed from reactions of wild-type eggs. (*E*) Localization of GM3 and CD9 in CD9⁺/⁺, CD9⁻/⁻, and Tg⁺CD9⁻/⁻ eggs. (*Left*) Wild-type. (*Middle*) CD9⁻/⁻. (*Right*) Tg⁺CD9⁻/⁻ eggs. Right-side of the sets of wild-type and Tg⁺CD9⁻/⁻ eggs are enlarged images of the boxed regions. The live eggs were examined, and the internal localization of GM3 in CD9⁻/⁻ eggs was examined under fixed, permeabilized conditions. (*F*) Comparison of the fluorescent intensities of GM3 stained by antibody in wild-type (*n* = 10), CD9⁻/⁻ (*n* = 9), and Tg⁺CD9⁻/⁻ eggs (*n* = 10) (mean ± SEM). The average values of the wild-type eggs were set to 100%.
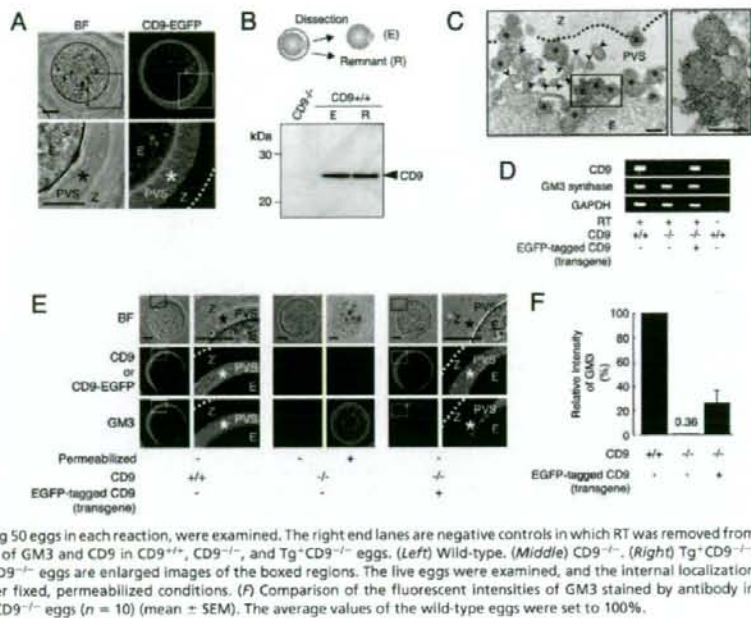
Tg⁺CD9⁺/⁻ and CD9⁺/⁻ females (6.0 ± 0.2 and 6.5 ± 0.5) and greater than those from CD9⁻/⁻ females (0.0 ± 0.0). The CD9⁺/⁻ females did not exhibit any loss in fertility that could cause a reduction of litter size relative to that of the CD9⁺/⁺ females (4). Furthermore, the transgene had no effect on normal fertility. These results demonstrate that transgenically expressed CD9-EGFP can compensate for the loss of intrinsic CD9 and yield eggs with the ability to fuse with sperm.

Based on the foregoing evidence, we observed the subcellular localization of CD9-EGFP in "living" Tg⁺CD9⁻/⁻ eggs (Fig. 2*A*). As expected, confocal microscopic analysis allowed the visualization of two types of CD9-EGFP localization: intense on the plasma membrane and also in the cytoplasm. Unexpectedly, we found loosely filled, noncompacted CD9-EGFP in the PVS, a space formed between the zona pellucida and the plasma membrane of the egg. The localization of CD9 outside the eggs also was confirmed by Western blot analysis using anti-CD9 mAb (Fig. 2*B*). As shown in the diagram, CD9⁺/⁺ eggs were mechanically fractionated into denuded eggs and other components (R) using a piezo manipulator (11). The fraction R, containing the zona pellucida and the components in the PVS, was centrifuged and subjected to Western blot analysis. The amount of CD9 in the remnant material from 40 eggs was found to be densitometrically equal to that of 10 zona-free eggs, demonstrating an estimated relative abundance of CD9 in the remnant of 20% per egg. Subsequently, we performed immunoelectron-microscopic analysis on the CD9⁺/⁺ eggs. We identified the vesicles bound to gold particles inside the PVS (Fig. 2*C*). The sectioned microvilli contained a branched network of actin filaments, whereas the variously sized vesicles (50–250 nm in diameter) had uniformly dense materials rather than actin filaments. We also compared CD9⁺/⁺, Tg⁺CD9⁻/⁻, and CD9⁻/⁻ eggs by electron-microscopic analysis [supporting information (SI) Fig. S1]. The accumulation of vesicles in the PVS in the Tg⁺CD9⁻/⁻ eggs was comparable to that in the CD9⁺/⁺ eggs, whereas it was not seen in the CD9⁻/⁻ or germinal vesicle-staged CD9⁺/⁺ eggs. These results indicate

that 20% of the total amount of CD9 is stored as vesicles in the PVS during meiosis.

We next examined the expression of ganglioside GM3, identified as a CD9-associated molecule (12) and a component of exosomes (10), in CD9⁺/⁺, CD9⁻/⁻, and Tg⁺CD9⁻/⁻ eggs. First, we confirmed the expression of GM3 synthase (ST3GalV/SAT-1) (13) in these eggs by RT-PCR (Fig. 2*D*). Then we investigated the localization of GM3 by immunostaining these live eggs with anti-GM3 mAb (Fig. 2*E*). This antibody has been demonstrated to recognize GM3 in the plasma membrane of cells without treatment for permeabilization (14). Finally, we measured the fluorescent intensities of GM3 in these live eggs (Fig. 2*F*). As expected, in wild-type eggs, GM3 was colocalized with CD9 in the PVS and plasma membrane (Fig. 2*E Left* and Fig. 2*F*). In contrast, in CD9⁻/⁻ eggs, the fluorescent intensities of GM3 were decreased dramatically in the PVS and plasma membrane (0.4% ± 0.2%, relative to 100% for the CD9⁺/⁺ eggs), consistent with the loss of CD9 (Fig. 2*E Center* and Fig. 2*F*), whereas GM3 could be detected in the cytoplasm of CD9⁻/⁻ eggs that had been permeabilized by a detergent after fixation. Moreover, the expression of CD9-EGFP reversed the decrease of GM3 in the PVS and plasma membrane of CD9⁻/⁻ eggs (25.6 ± 10.7%) (Fig. 2*E Right* and Fig. 2*F*), corresponding to the amount of CD9-EGFP quantified by Western blot analysis (Fig. 1*B*). In addition, electron-microscopic analysis revealed that the number of characteristic membrane structures, termed microvilli (1), were significantly decreased in the CD9⁻/⁻ eggs compared with the CD9⁺/⁺ eggs (Fig. S2 *A* and *B*). The numbers of microvilli were increased by ~50% by the expression of CD9-EGFP in the CD9⁻/⁻ eggs. The analyses of three types of eggs indicate that CD9- and GM3-containing vesicle release is linked to microvilli formation.

We next investigated the involvement of CD9-containing vesicles in sperm–egg fusion (Fig. 3). We found that, based on the length of microvilli (Fig. S2*C*), zona-intact Tg⁺CD9⁻/⁻ eggs can be categorized into two groups (Fig. 3*A*). From single
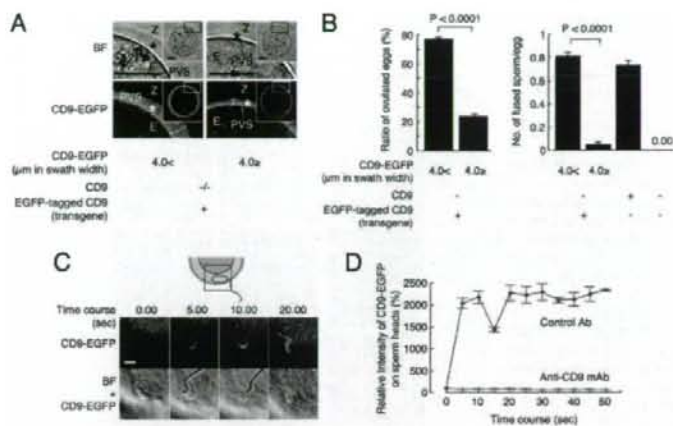
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Fig. 3. Involvement of CD9-containing vesicles in sperm–egg fusion. (*A*) Categorization of Tg$^+$CD9$^{-/-}$ eggs (E) into two groups according to the thickness of CD9-EGFP in the PVS (*) and the inner region of the zona pellucida (Z) (>4.0 μm or $\leq$ 4.0 μm), indicated by double-headed lines. The boxed regions in *Insets* are enlarged. Scale bar: 20 μm. (*B*) Comparison of the fusing ability of two groups of Tg$^+$CD9$^{-/-}$ eggs with wild-type sperm. Left graph: Ratio of two groups of Tg$^+$CD9$^{-/-}$ eggs ovulated from 12 females (mean ± SEM). Right graph: Number of sperm fused per egg in two groups of zona-intact Tg$^+$CD9$^{-/-}$ eggs ovulated from 12 females (>4.0 μm, *n* = 204; $\leq$4.0 μm, *n* = 66) (mean ± SEM). CD9$^{+/+}$ (*n* = 120) and CD9$^{-/-}$ (*n* = 112) served as positive and negative controls, respectively. (*C* and *D*) Monitoring of the association of egg CD9-containing vesicles with wild-type sperm. Tg$^+$CD9$^{-/-}$ eggs were incubated with the sperm and monitored immediately after the sperm penetrated the zona pellucida under the presence of anti-CD9 mAb (boxed region). The values were calculated from data scanning by confocal microscopy (15 sperm in triplicate dishes) Blue: Preimmune rat IgG. Red: Anti-CD9 mAb (KMC8) (mean ± SEM). The average values of the fluorescent intensities of CD9-EGFP at 0 s were set to 100%, and the final concentration of antibodies was adjusted to 50 μg/ml. Scale bar, 5 μm.

confocal images sectioned through the largest diameter, the accumulation of CD9-EGFP from the plasma membrane to the inner region of the zona pellucida was >4.0 μm in swath width in one group and $\leq$ 4.0 μm in the other group. The accumulation of CD9-EGFP was predicted to show that CD9-containing vesicles are more highly accumulated within the PVS in the >4.0-μm group compared with the $\leq$ 4.0-μm group. Comparing the ratio of these two groups in Tg$^+$CD9$^{-/-}$-ovulated eggs revealed a much higher percentage of the >4.0-μm group (77.0 ± 1.3% vs.23.7 ± 1.5%) (Fig. 3*B Left*). Therefore, we focused on the heterogeneity of CD9-EGFP accumulation within the PVS and determined the ratio of the two groups in zona-intact Tg$^+$CD9$^{-/-}$ eggs that successfully fused with the sperm 6 h after insemination. The >4.0-μm group of Tg$^+$CD9$^{-/-}$ eggs showed higher activity for fusion with sperm (0.81 ± 0.04 sperm fused per egg), compared with the $\leq$40-μm group of Tg$^+$CD9$^{-/-}$ eggs (0.05 ± 0.03) and the CD9$^{-/-}$ eggs (0.00 ± 0.00) (Fig. 3*B Right*). The average activity of all Tg$^+$CD9$^{-/-}$ eggs (0.72 ± 0.03 sperm fused per egg) was equal to that of wild-type eggs (0.73 ± 0.04 sperm fused per egg). The difference between the two groups of Tg$^+$CD9$^{-/-}$ eggs was statistically significant (Fig. 3*B*). These results suggest that the quantities of CD9-containing vesicles, as assessed by the swath width of CD9-EGFP, are strongly correlated with the frequency of sperm–egg fusion.

To detect the association between sperm and CD9-containing vesicles, we serially monitored the wild-type sperm that penetrated the zona pellucida of the Tg$^+$CD9$^{-/-}$ eggs (Fig. 3 *C* and *D*). As shown in the diagram, we began monitoring the sperm immediately after the head portion of sperm penetrated the zona pellucida of the Tg$^+$CD9$^{-/-}$ eggs (Fig. 3*C Upper*, boxed area in the diagram). Soon after we began to monitor the sperm, the fluorescent intensities of CD9-EGFP on the sperm heads increased and then decreased rapidly between 0 s and 15 s, then increased again, reaching a maximum at 20 s. At this point, the

CD9-EGFP fully covered the surface of the sperm heads. In contrast, when the sperm were incubated with Tg$^+$CD9$^{-/-}$ eggs in the medium containing anti-CD9 mAb, no increase in intensity of CD9-EGFP on the sperm heads was detected. Anti-CD9 mAbs have been reported to inhibit sperm–egg fusion (4, 15, 16). Our findings demonstrate that the anti-CD9 mAb inhibited the association of sperm with CD9-containing vesicles in parallel to inhibition of sperm–egg fusion.

To determine whether CD9-containing vesicles are capable of initiating sperm–egg fusion, we incubated the sperm with CD9$^{-/-}$ eggs in medium containing the vesicles collected from CD9$^{+/+}$ eggs (Fig. 4 and Fig. S3). To restrict the source of CD9 into the vesicles from the CD9$^{+/+}$ eggs, we used sperm collected from the epididymis of CD9$^{-/-}$ males. We estimated the capability of the vesicles to influence fusion by counting the number of sperm fused with CD9$^{-/-}$ eggs. As shown in the experimental design, after the zona pellucida was removed from the CD9$^{-/-}$ eggs, the eggs were incubated with sperm in the medium containing the vesicles (Fig. 4*A*). When examined at 1 h after incubation, the sperm were seen to be capable of fusing with CD9$^{-/-}$ eggs after co-incubation with the vesicles (Fig. 4*A Center*), indicating restoration of the fusibility of CD9$^{-/-}$ eggs with the sperm (0.58 ± 0.07 sperm fused per egg) (Fig. 4*B*). We detected further evidence of sperm–egg fusion in the CD9$^{-/-}$ eggs from which a second polar body had been extruded. In contrast, we did not detect improved fusibility of sperm with eggs in medium depleted of CD9-containing vesicles using beads conjugated with anti-CD9 mAb (Fig. 4*A Right* and *B*). After treatment with the beads, the quantity of CD9 in the depleted medium was significantly decreased, to 16% of the untreated medium (Fig. 4*C*). In addition, CD9$^{-/-}$ remnants failed to rescue the fusing ability of CD9$^{-/-}$ eggs. These findings indicate that the association with CD9-containing vesicles renders the sperm capable of fusing with eggs without endogenous CD9 expression. We estimated the relative abundance of CD9 in the remnant as 18% of the total amount in the eggs (Fig. 4*C*). We further found

Fig. 4. Identification of fusion-facilitating activity of CD9-containing vesicles. (A) Estimation of the fusion-facilitating ability of the vesicles in sperm–egg fusion. As shown in the experimental design, CD9$^{-/-}$ sperm were incubated with CD9$^{-/-}$ eggs (white circles) in media containing egg-released vesicles after the zona pellucida was removed from these eggs. CD9 was detected by anti-CD9 mAb conjugated with Alexa488. The eggs were preloaded with DAPI before incubation with the sperm, to allow counting of the number of fused sperm. (Left) CD9$^{-/-}$ eggs at 1 h after incubation with the sperm, as a negative control. Center CD9$^{-/-}$ eggs cultured in the medium containing CD9 collected from wild-type eggs. (Right) CD9$^{-/-}$ eggs cultured in the medium depleted of CD9 by beads conjugated with anti-CD9 mAb, showing the fused sperm to eggs (arrowhead), metaphase II–arrested chromosomes (*), a second polar body (open arrowhead), and CD9 translocated on the sperm heads (arrow). The fluorescent z-series images were projected as three-dimensional images. Scale bar: 20 μm. (B) Number of fused sperm with the zona-free eggs counted at 1 h after incubation (mean ± SEM): CD9$^{-/-}$ eggs as a negative control (n = 51), CD9$^{-/-}$ eggs cultured in the medium containing CD9 (n = 112), and CD9$^{-/-}$ eggs cultured in the medium depleted of CD9 by antibody-conjugated beads (n = 74). The total numbers of eggs examined are in parentheses. (C) Western blot analysis of the media incubated with CD9$^{+/+}$ eggs for CD9, HSP90, and HSP60. Loaded samples (left to right): The medium as a negative control, the medium containing the remnant material from 40 eggs per lane (bead-untreated and -treated), and 5 eggs per lane as a positive control. The albumin contained in the medium was detected by Coomassie brilliant blue staining as an internal control. The quantities of CD9 in the media were measured densitometrically (using National Institutes of Health Image software).
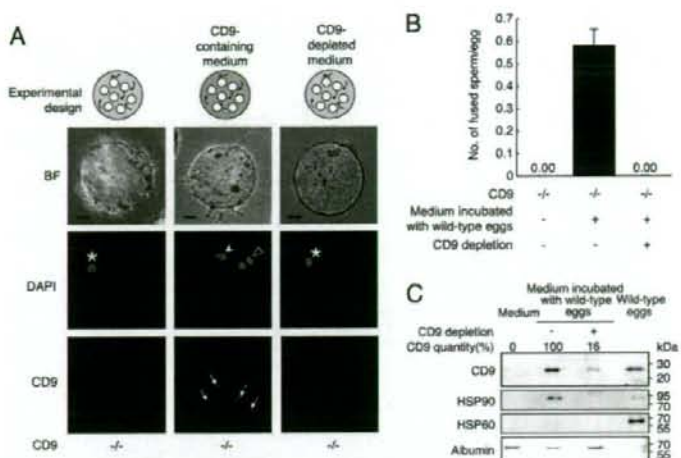
that the decreased amount of CD9 after the bead treatment was synchronized with that of a cytoplasmic chaperone, HSP90 (17), but not with a mitochondrial chaperone, HSP60 (18). Our analysis of the egg-conditioned medium indicated that CD9-containing vesicles contained HSP90, a conserved component of exosomes (9, 10).

To estimate the contribution of CD9-containing vesicles to sperm–egg fusion, we examined the restoration of the impaired sperm-fusing ability in CD9$^{-/-}$ eggs co-incubated with CD9$^{+/-}$ or CD9$^{+/+}$ eggs expressing endogenous CD9 (Figs. 5 and S4.4). We predicted that when sperm were incubated with a mixture of eggs, the vesicles released from CD9$^{+/-}$ or CD9$^{+/+}$ eggs would

Fig. 5. Recovery of impaired fusion of CD9$^{-/-}$ eggs with sperm by CD9-containing vesicles. (A) Estimation of the fusion-facilitating ability of the vesicles in sperm–egg fusion. As shown in the experimental design, sperm were incubated with a mixture of CD9-expressing eggs (green circles) and CD9$^{-/-}$ eggs (red circles) after the zona pellucida was removed from these eggs. The eggs were preloaded with DAPI before incubation with sperm, to allow counting of the number of fused sperm. CD9$^{-/-}$ eggs were prestained with FM4–64 and thus were easily distinguished from CD9-expressing eggs after incubation with the sperm. (Left) CD9$^{+/-}$ eggs at 1 h after incubation with the sperm, as a positive control. (Center) CD9$^{-/-}$ eggs, as a negative control. (Right) CD9$^{-/-}$ eggs co-incubated with CD9$^{+/-}$ eggs, showing fused sperm to egg (arrowheads), metaphase II–arrested chromosomes (*), and extruded second polar body (arrow). The fluorescent z-series images were projected as three-dimensional images. CD9 was detected by anti-CD9 mAb conjugated with Alexa488. Scale bar: 20 μm. (B) Numbers of fused sperm with the zona-free eggs counted at 1 and 3 h after incubation (mean ± SEM). CD9$^{+/-}$ (1 h: n = 34; 3 h: n = 55), CD9$^{+/+}$ (1 h: n = 71; 3 h: n = 79), and CD9$^{-/-}$ eggs (1 h: n = 100; 3 h: n = 115) were separately incubated with sperm. Total number of coincubated eggs examined: CD9$^{+/-}$ eggs (n = 54) coincubated with CD9$^{-/-}$ eggs (n = 60), and CD9$^{+/+}$ eggs (n = 65) coincubated with CD9$^{-/-}$ eggs (n = 74) at 1 h; CD9$^{+/-}$ eggs (n = 51) coincubated with CD9$^{-/-}$ eggs (n = 33), and CD9$^{+/+}$ eggs (n = 98) coincubated with CD9$^{-/-}$ eggs (n = 90) at 3 h. (C) Schematic model of involvement of CD9-containing vesicles in sperm–egg fusion: CD9$^{+/+}$ (green), CD9$^{-/-}$ (light blue), and CD9$^{-/-}$ eggs coincubated with CD9$^{+/+}$ wild-type eggs with sperm (yellow).
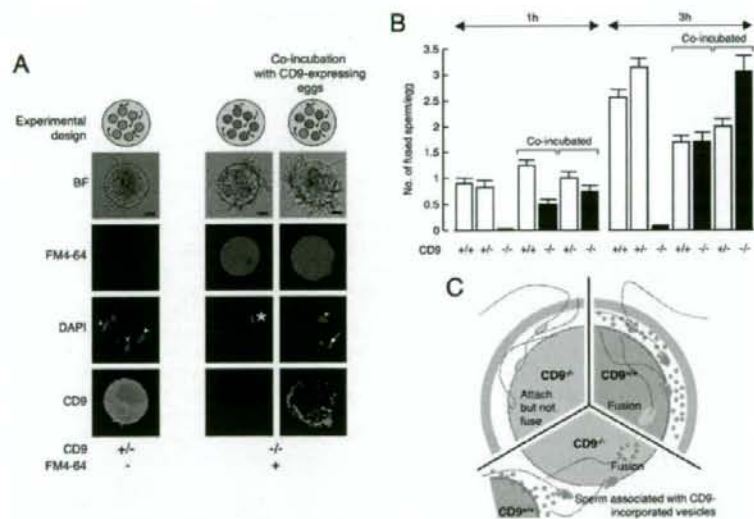
interact with sperm, and these sperm could fuse with CD9$^{-/-}$ eggs. If sperm-fusing ability were regulated mainly by CD9-containing vesicles, then the number of sperm fused to CD9$^{-/-}$ eggs would be predicted to be almost equal to that fused to CD9$^{+/-}$ or CD9$^{+/+}$ eggs coincubated with CD9$^{-/-}$ eggs. We counted the number of fused sperm in coincubated CD9-expressing eggs (CD9$^{+/-}$ and CD9$^{+/+}$) and CD9$^{-/-}$ eggs. The CD9$^{-/-}$ eggs were prestained with FM4–64 (19), a fluorescent dye used to stain the membrane of live cells, and thus could be easily distinguished from the CD9$^{+/-}$ and CD9$^{+/+}$ eggs. FM4–64 did not transfer between the CD9$^{-/-}$ eggs and the CD9$^{+/-}$ or CD9$^{+/+}$ eggs. As shown in the experimental design, after the zona pellucida was removed from the eggs, CD9$^{-/-}$ eggs (red circles) were mixed with CD9$^{-/-}$ or CD9$^{+/+}$ eggs (green circles), and sperm were added to the medium containing these eggs (Fig. 5A). At 1 h after insemination, significant fusion of sperm with the CD9$^{-/-}$ eggs was facilitated (0.75 ± 0.11 and 0.50 ± 0.09 sperm fused per egg), corresponding to that in the CD9$^{+/-}$ (1.00 ± 0.13) and CD9$^{+/-}$ eggs (1.25 ± 0.10). At 3 h after insemination, the fusion of sperm with the CD9$^{-/-}$ eggs was restored (3.06 ± 0.30 and 1.70 ± 0.18 sperm fused per egg) to levels comparable to those in the CD9$^{+/-}$ (2.00 ± 0.15) and CD9$^{+/+}$ eggs (1.69 ± 0.13). We also detected a second polar body extruding from the CD9$^{-/-}$ eggs (Fig. 5A Right, arrow). In contrast, we did not observe the translocation of vesicles from the CD9$^{+/-}$ and CD9$^{+/-}$ eggs to the CD9$^{-/-}$ eggs when sperm were not added to the mixture, even after 10 h of incubation (Fig. S4B). These data demonstrate that the defect in the fusing ability of CD9$^{-/-}$ eggs is caused by dysfunction of the mechanism facilitating the sperm-fusing activity through CD9-containing vesicles.

To further study the involvement of CD9-containing vesicles in regulating sperm-fusing ability, we evaluated the capability of hamster eggs in sperm–egg fusion (Fig. S5). Hamster eggs have the ability to fuse with other mammalian sperm and thus are used as a tool to evaluate the fusing ability of human sperm (20). When hamster eggs were incubated with CD9$^{-/-}$ eggs after the zona pellucida was removed from these eggs, the sperm-fusing ability of these eggs was improved significantly. The sperm-fusing ability acquired through the exposure to hamster eggs was not as great as that produced by exposure to mouse eggs, probably due to the slightly different CD9 in hamster and mouse eggs (21). These results indicate that the function of CD9-containing vesicles in the acquisition of sperm-fusing ability is widely conserved in mammals.

Discussion

In sperm–egg fusion, there is a significant direct interaction between the cell membranes of sperm and eggs (1, 20, 22); however, our results demonstrate that CD9-containing vesicle–sperm interaction precedes the direct cell membrane interaction between sperm and eggs. Based on our data, we propose that the release of CD9-containing vesicles from eggs before fertilization facilitates the sperm-fusing ability that renders the sperm competent to fuse with CD9$^{-/-}$ eggs (Fig. 5C). Our finding of CD9-EGFP in living unfertilized eggs demonstrates that CD9-containing vesicles are present in the PVS, and that these vesicles accumulate inside the PVS during the germinal vesicle (1) and metaphase II-arrested stages (1). During this period, the egg undergoes drastic cytological changes with the increased number of microvilli (1, 22), predicting the correlation between vesicle release and microvilli formation. As expected, this correlation is supported by the finding that CD9 deficiency leads not only to impaired microvilli formation (8) (Fig. S2), but also to decreased accumulation of vesicles within the PVS. These data support the association between the release of CD9-containing vesicles from eggs and the formation of microvilli on the egg plasma membrane.

As reported previously, somatic cells are capable of releasing proteins and lipids included in membrane organelles, termed exosomes (9, 10), which are pinched out from the plasma membrane (23). Exosomes share many additional properties with retroviral particles, including similar lipid and protein compositions, such as tetraspanin (23). GM3 and HSP90 are known to be conserved components of exosomes (10). Our results show that CD9-containing vesicles released from eggs share these two components, implying that the vesicles are "exosome-like." Previous studies of macrophages have proposed that exosome biogenesis occurs only by outward budding at endosomal membranes, followed by the fusion of vesicle-laden endosomes with the plasma membrane (9, 23). If the CD9-containing vesicle were derived from exosomes and generated from the fusion of endosomes with the plasma membrane, then the vesicles would contain some proteases (9, 23), fuse with the sperm membrane, and possibly activate the sperm fusogenic factor(s) by enzymatic activities.

In hamster eggs, expansion of the PVS has been deemed essential or at least beneficial to normal fertilization (20, 21, 24), indicating that materials involved in fusion with sperm are released from eggs before fertilization in hamsters and in mice. Because anti-CD9 mAbs are not available for hamster CD9, we could not directly confirm CD9-containing vesicle release from hamster eggs before fertilization. Instead, our co-incubation assay demonstrated that hamster eggs facilitate the fusion of sperm with CD9$^{-/-}$ eggs, indicating that hamster eggs share a similar mechanism with mouse eggs through egg-released materials. Moreover, it has been reported that growing oocytes bind to sperm and transfer fluorescent dyes to the sperm head (25). At this stage, oocytes have CD9 on the cell membrane but lack CD9-containing vesicles (Fig. S1). We presume that the transfer of fluorescent dye from growing oocytes to sperm heads is mediated by CD9 on the cell membrane. Based on our findings, we propose that the CD9-containing vesicle has an ability to facilitate sperm—egg fusion. This knowledge has great potential for clinical applications, such as the induction of sperm–egg fusion using exogenous sources.

Materials and Methods

Animals. The mice that we produced were back-crossed into a C57BL/6 genetic background. Wild-type eggs were collected from C57BL/6 females (8–12 weeks old). Wild-type sperm were obtained from the epididymides of B6C3F1 males (8–12 weeks old). Hamster eggs were obtained commercially as frozen unfertilized eggs (NOSAN).

Antibodies and Chemicals. Antibodies against CD9 (KMC; BD PharMingen), beta-tubulin (Tub2.1; Sigma), HSP60 (24/HSP60; BD PharMingen), HSP90 (16F1; MBL), and GM3 (GMR6; Seikagaku) were used. Antibodies labeled with biotin by a labeling kit (Dojindo) and horseradish peroxidase-conjugated streptavidin (Sigma) were used for Western blot analysis. For immunostaining, antibodies were labeled directly with Alexa488 and Alexa546 using labeling kits (Invitrogen). FM4–64 (Invitrogen) was used to define the lipid bilayer of live eggs without disturbing sperm–egg fusion (10 μM at final concentration). We used DAPI (Invitrogen), a fluorescent dye that slowly permeates the living cell membrane (semipermeable) and slowly leaks out of cells after washing relative to Hoechst33342 (permeable), in counting the number of sperm fused per egg.

Transgenic Mice. The construct expressing mouse CD9 tagged at the N terminus with EGFP (CD9-EGFP) was subcloned into plasmid DNA-containing mouse ZP3 promoter (26). The expression cassette was excised by restriction enzyme digestion and microinjected into fertilized eggs of C57BL/6 mice, according to standard techniques (27).

Genotyping and RT-PCR. Mouse genotyping and RT-PCR were performed following standard procedures (27). (Primer sets are listed in Table S1).

Egg Collection. Eggs were collected from the oviduct 14–16 h after human chorionic gonadotropin injection (4). The eggs were placed in a drop of TYH

medium (28). Sperm collected from the epididymides were capacitated in a 100-μl drop of medium. The eggs were incubated with 1.5×10^5 sperm/ml at 37°C in 5% CO_2, and unbound sperm were washed away. The zona pellucida was removed from the eggs with acidic Tyrode's solution (4) or a piezo manipulator (11). A hole was punched through the zona pellucida with a piezo manipulator, and the eggs were removed. All materials were aspirated, including the medium but not the eggs, and used as "remnants."

Immunostaining. Zona-intact live eggs were stained with diluted antibodies in TYH medium for 30 min at 37°C, and the nonspecifically accumulated antibodies in the PVS were washed away after a brief incubation (30 min) in the medium. To measure the fluorescent intensities of GM3, three types of eggs were stained by Alexa546-labeled anti-GM3 mAb in TYH medium for 30 min, then washed in the medium for 30 min. Staining was visualized using a laser scanning confocal microscope (LSM 510 META; Carl Zeiss).

Electron-Microscopic Analysis. Live eggs were incubated with anti-CD9 mAb and anti-rat IgG mAb tagged with 5-nm gold beads. After incubation, the eggs were fixed by glutaraldehyde and osmic acid solutions. Ultra-thin sections were prepared as described in ref. 29. Eggs denuded with acid Tyrode's solution were fixed with a mixture of paraformaldehyde and glutaraldehyde and osmic acid solutions.

In Vitro Fertilization. To observe the fusion with the sperm, zona-intact and zona-free eggs were incubated with DAPI (10 μg/ml) in the medium for 20 min, then washed before the sperm were added. This procedure allowed the staining of only fused sperm nuclei by dye-transfer into sperm after membrane fusion. At 1 h or 3 h after incubation in a 30-μl drop of medium, the eggs were fixed with a mixture of paraformaldehyde and glutaraldehyde for 20 min at 4°C.

Monitoring the Association of CD9-Containing Vesicles with Sperm. Eggs collected from Tg$^+$CD9$^{-/-}$ females were set in a 30-μl drop of TYM medium. The sperm were added to the eggs at a final concentration of 1.5×10^5/ml after incubation in the medium for 2 h. Posts of latex beads were deposited around the eggs. A glass coverslip was carefully pressed down onto the posts until the egg were fixed. The medium containing eggs and sperm was cooled to 10°C

before observation. Cooling reduced the sperm motility. This procedure allowed us to measure the CD9-EGFP fluorescence on the sperm head using a confocal microscope. Images of the sperm were captured at 1 frame/s. The average value of the fluorescent intensities of CD9-EGFP at 0 s was set to 100%, and the final concentration of antibodies was adjusted to 50 μg/ml. The data are measurements of serial images from 15 wild-type sperm in triplicate dishes.

Collection of CD9-Containing Vesicles. The medium containing the vesicles was collected from denuded wild-type eggs. The eggs were cultured in a 60-μl drop of medium for 2 h after the zona pellucida was removed from the eggs. Collecting the medium containing the vesicles required an incubation time of 2 h. The collected medium was used for analysis of vesicle components and evaluation of sperm-fusing ability. CD9-depleted medium was used as a negative control. After the zona pellucida was removed from CD9$^{-/-}$ eggs, the eggs were incubated with the sperm in the medium containing CD9-incorporated vesicles for 1 h, for comparison with the vesicle-depleted medium. Details are shown in Fig. S3.

Western Blot Analysis. Quantities of proteins were examined by Western blot analysis, as described in ref. 4. As an internal loading control, quantities of albumin included in the medium were examined using Coomassie brilliant blue staining. Details are shown in Fig. S3.

Coincubation of Two Types of Eggs. CD9$^{-/-}$ eggs and CD9-expressing eggs (CD9$^{+/-}$ and CD9$^{-/+}$) were incubated in each 30-μl drop of medium after the zona pellucida was removed from these eggs. At 2 h after incubation, the CD9$^{-/-}$ eggs were added into the cultured medium of the CD9-expressing eggs. Sperm were added into the medium containing two types of eggs and incubated for 1 or 3 h. Details are shown in Fig. S4A. The frozen hamster eggs also were incubated with the CD9$^{-/-}$ eggs and wild-type sperm for 1 h. The zona pellucida of frozen hamster eggs was hardened, and removing the zona pellucida using acid Tyrode's solution took 5 min. Details are shown in Fig. S5A.

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