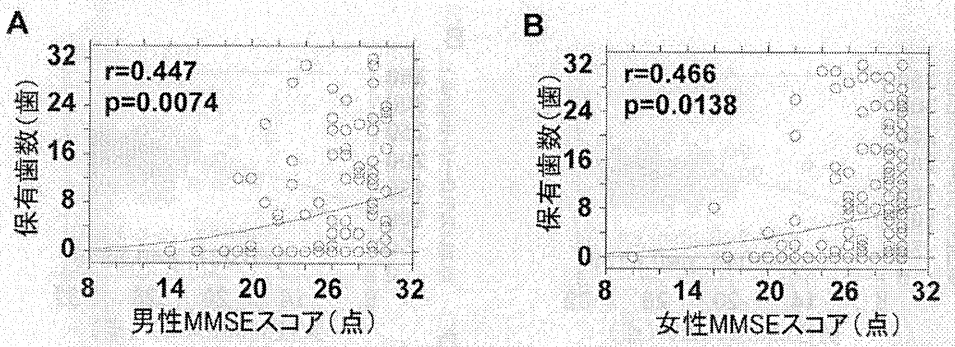

図5. 保有歯数と認知機能の相関関係
65－92歳の男女についてMMSEテスト，保有歯数の検定を行った。MMSEスコアと保有歯数の相関関係はノンパラメトリックのスペアマン相関分析法にて解析した。相関係数r，p値を各々グラフ内に表記した。男女とも，MMSEと保有歯数との正の相関関係がみられた（$p<0.05$，$r>0.4$）。

今回の解析結果では，高齢者の血清脂質と認知機能には有意な相関がみられなかった．その理由として，今回の被験者に血清脂質の数値が高い者がほとんど認められず，ほとんどの者が正常範囲内であったこと，高年期における血清脂質の異常は，認知機能低下のリスクにならない可能性などが考えられる．一方，中年期における高脂血症や肥満の有無は認知機能に影響を与えた可能性は考えられるが，それらの既往については調査されなかったため，過去の病歴との関連性を調べることはできなかった．糖尿病と認知機能との相関についても報告があり，特に2型糖尿病との関連性が指摘されている[33,34]．加えて，中年期の糖尿病患者は将来的な認知機能低下のリスクが高いことが示されている[35,36]．今回の解析結果では，血清脂質の結果と同様に糖代謝マーカーと認知機能との間に有意な相関関係は認められなかった．従って，高年期における糖代謝異常は認知機能に影響を及ぼしていない可能性が考えられた．

歯の保有歯数あるいは機能歯の数と認知機能との間には有意な相関関係があることが報告されている[11,37,38]．本解析結果においても，被験高齢者の保有歯数とMMSEスコアとの間に有意な正の相関がみられた．本調査では検査項目が多岐にわたっていたことや時間の制約があったことから，口腔内の検査は保有歯数に限られており，齲蝕歯や健全歯の区別，義歯の有無，歯周病の有無等の検査が行われていない．従って，口腔疾患や口腔機能等との総合的な相関はみることができなかった．しかし，血液の各種指標と認知機能との相関はみられなかったにもかかわらず，認知機能と保有歯数との間に相関関係がみられたことは極めて興味深い．高齢者の歯数は，高齢者の認知機能を推し量る，簡便なサロゲートマーカーとなり得る可能性がある．認知症患者あるいは認知機能が低下した高齢者は歯科を受診する機会が少なく，また自宅や施設における口腔清掃状態も不良であるケースが多くなるため，歯を喪失しやすくなることが考えられる．加えて，歯の喪失は咀嚼機能の低下に繋がり，それは脳血流の低下を引き起こして，認知機能の低下を助長するかもしれない．また，歯の喪失の大きな原因として歯周病や齲蝕が考えられるが，それらは食習慣をはじめとした生活習慣が深く関わる疾患である．特に，歯周病は糖尿病，肥満，心血管病等の生活習慣病との相関関係が指摘されている[39,40]．従って，歯の喪失の背景には，生活習慣の問題が隠されており，それが認知機能の低下に直接あるいは間接的に関与した可能性も考えられる[41,42]．本研究の結果は，歯を残すことが単に口腔のQOLの維持に重要であるばかりでなく，健やかに老いるための重要な要素となっていることを示唆しているものと考える．

V. 結　　論

高知県香北町の高齢者を対象とした疫学調査について解析した結果，認知機能と脂質・糖質代謝パラメータとの間に有意な相関関係は認められなかったが，認知機能と保有歯数との間には相関関係があることが明らかになった．

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高齢者の口腔・歯科疾患と免疫能

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はじめに

高齢者は慢性疾患の罹患率が極めて高く、また複数の疾患を罹患しやすいといった特徴を有している。また、同じ疾患においても高齢者では成人と異なった症状を呈することも多い。加えて、生体防御力が低下しており、疾患が治りにくいといった特徴がある。口腔領域においても高齢者においては慢性疾患が多発する傾向があり、高齢者に特有の症状を呈する場合も少なくない。高齢者では感染症が増加し、特に誤嚥性などの肺炎による死者が多くなるが、その原因の1つとして加齢に伴う免疫機能の低下が考えられる。

加齢による免疫系の変化は、高齢者における感染症、自己免疫疾患、ガンの発症の増加等に深く関与している。免疫系の加齢変化は、骨髄、胸腺、末梢血、あるいは2次リンパ節の種々の細胞、あるいは自然免疫系の様々な因子に影響を及ぼす。老化は、自然免疫系、獲得免疫系どちらの系に対しても影響するが、特に獲得免疫系が影響を受けると考えられる。本稿では、高齢者に特有の口腔疾患と免疫との関連について考察したいと思う。

自然免疫系の加齢変化

自然免疫系は生体防御の第一線として働き、感染防御のための重要な生体システムである。老化は皮膚、肺、消化管などの上皮のバリアー機能の低下を引き起こし、病原体の体内への侵入を容易にする[1,2]。自然免疫系で重要な液性因子、例えばinterleukin (IL)-6, IL-1

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β, tumor necrosis factor-αなどのサイトカインが高齢者の血清中では上昇していることが報告されている[3]。また、高齢者は、様々な基礎疾患を有していたり、また細菌の侵襲を受けやすかったりし、それが症状としては現れない炎症反応を持続的に惹起しているため、血中においてそのような炎症マーカーが検出される可能性が考えられる。加齢による自然免疫系の変化をTable 1にまとめた。好中球は急性炎症期の細菌や真菌感染を防御する重要な細胞である。好中球数については、成人も高齢者も大差がない[4]。

また、高齢者の好中球も十分な走化性を示す。その一方、高齢者の白血球はオプソニン化された細菌の貪食能が劣ること[5]、またFcレセプターを介した活性酸素産生が低下していること[6]などが報告されている。また、マクロファージに関しても、高齢者においては成人に比べて細菌の貪食能や活性酸素産生による殺菌能が低下していることが明らかになっている[7、8]。樹状細胞に関しては、抗原特異的T細胞を活性化するための抗原提示能、具体的には、抗原貪食能やリンパ節へのホーミング能が低下している[9、10]。natural killer（NK）細胞はウイルス感染細胞やがん細胞の排除に働く細胞であるが、高齢者において同細胞の増加が認められる[11、12]。しかしながら、その機能は低下しており、感染細胞に対する障害活性やIFN－γ産生能は高齢者において低下している[13]。

獲得免疫系の加齢変化

獲得免疫は、自然免疫をくぐり抜けてきた病原体や癌細胞等に対する特異免疫を増幅し、効率的にそれらを排除する生体防御機構であるが、老化によってこの機構は大きく変調をきたす。加齢による獲得免疫系の変化を表2にまとめた。獲得免疫系における重要な細胞であるT細胞は胸腺で産生される。胸腺は加

表1　加齢による自然免疫系の変化

細胞種	増加	減少
好中球		活性酸素産生 貪食能 殺菌能
マクロファージ		活性酸素産生 貪食能
NK細胞	細胞数	増殖能 IL-2に対する反応性 細胞障害活性
樹状細胞		抗原提示能 ホーミング活性
サイトカイン	血清中のIL-6, IL-1β, TNF-α	

齢によって萎縮してゆき、40～50歳頃には殆どなくなってしまう[14]。その結果、胸腺由来のナイーブT細胞は、劇的に減少してしまう[15]。樹状細胞の機能低下と相まって、T細胞の減少は抗原特異的T細胞の増幅が劇的に減少する[16~18]。さらに、IL－2産生能等も低下するため、高齢者においては新規の抗原に対する十分な免疫応答が起こりにくくなっている[19~21]。さらに、胸腺の萎縮は、調節性T細胞（Tregs）の減少を引き起こす[22]。このことは、高齢者に特徴的な現象である炎症の増加や自己免疫疾患の増加と関連しているものと推察される。

抗体産生に重要な細胞であるB細胞も加齢による変化が見受けられる。ナイーブB細胞の減少によって、相対的にメモリー細胞の比率が高い傾向が高齢者にはみられる[23,24]。また、CD40やCD27といったB細胞上の共刺激分子の発現低下による抗体産生細胞への分化能の低下[25]、さらにはIgMからIgG産生へのスイッチの消失等が起こり[26]、老化によって体液性免疫応答の低下が起こると考えられる。このような老化による獲得免疫応答の低下あるいは異常が高齢者特有の口腔疾患の発症や病態形成に関わっている可能性が考えられる。

高齢者特有の口腔疾患と免疫

1）齲蝕

齲蝕は日本国民の殆どが罹患する疾患であるが、高齢者の齲蝕と成人の齲蝕とでは様相が異なっている。高齢者においては歯肉の退縮や歯周病の罹患により根面う蝕のリスクが飛躍的に高まる。60歳以上の根面う蝕の罹患率は30歳台のそれの約2倍となっている[27]。高齢者においては、根面の露出とともに唾液の減少、さらには齲蝕原性細菌に対する免疫応答の低下が相まって根面う蝕が進行しやすくなることがその原因として考えられる。

表2　加齢による獲得免疫系の変化

細胞種	増加	減少
T細胞	メモリー細胞とエフェクター細胞、エフェクター細胞のクローナルな増幅、炎症性サイトカインの産生	ナイーブT細胞数 T細胞レパトア 共刺激分子（CD28、CD27、CD40L等）の発現 調節性T細胞数（Tregs）
B細胞	自己抗体の産生	B前駆細胞数 ナイーブB細胞数 B細胞レパトア 共刺激分子（CD27、CD40等）の発現 IgGへのクラススイッチ

加えて、寝たきりの高齢者においては口腔清掃の不足も重要な要素となる。

2）歯周病

高齢者においては、歯肉退縮や口腔清掃不良が相まって歯周病に罹患している高齢者が多い。平均寿命の延伸と高齢者の残存歯数の増加によって、日本国の高齢者においては歯周病の罹患者は増加傾向にある。また、糖尿病の合併症でもあり、糖尿病に罹患した高齢者は、重度の歯周病に罹患しているケースが多い[28,29]。多くの疫学調査によって、歯周病と糖尿病の相関関係はある程度解明されたが、両者の因果関係を説明するにはまだ十分なエビデンスが蓄積されていないように思われる。糖尿病が歯周病を増悪する機序について、いくつかのことが想定される。その重要なファクターとして高血糖が挙げられる。高血糖は心血管疾患の主要なリスクファクターとして認知されている[30]が、高血糖は非酵素的に生体のタンパク質を糖化する。これらの糖化最終産物（AGE：Advanced glucation endoproducts）は、マクロファージをはじめとする炎症性細胞を活性化する[31]。AGEによって活性化された炎症性細胞は、動脈硬化巣へ集積し、TNF-α、IL-6、MCP-1などの炎症性サイトカインや活性酸素を産生し、血管の炎症を増悪して、動脈硬化を進展させる。AGEは、血管内皮細胞における一酸化窒素（NO）の産生を傷害し、血管内皮機能を低下させ、大小血管の循環障害を引き起こす[32]。また、糖尿病患者の歯周組織にはAGEが沈着していることが報告されている[33]。したがって、AGEによる動脈硬化の進展機序と同様にAGEによる全身性の炎症惹起と細小動脈を含む血管機能の障害によって、歯周組織の炎症の増悪と創傷治癒の遅延が起こっている可能性が考えられる（図1）。

また、歯周病が糖尿病を増悪する可能性も考えられる。歯周病は、歯周局所の感染症であり、歯周病病原細菌とそれに対する生体応答が全身に波及し糖尿病を増悪する可能性がある。歯周ポケットに形成される細菌性バイオフィルムは歯周局所に炎症反応を惹起し、歯周炎を引き起こす。その際、歯周局所には、炎症性サイトカインをはじめとする生理活性物質が多量に産生されるが、それは血行性に全身伝搬される。特にTNF-αはその受容体を介して骨格筋細胞や脂肪細胞による等の取り込みを抑制し、インシュリン抵抗性を付与する[34]。また、慢性の歯周炎患者においては、菌血症が頻繁に起こっていることが報告され

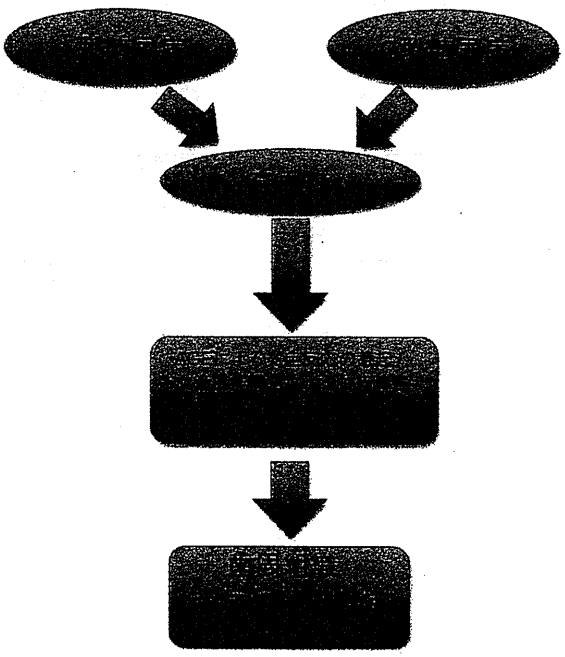

図1　糖尿病が歯周病に及ぼす影響の一例
糖尿病においてみられる高血糖は、炎症反応の増強、血管障害、代謝障害等を引き起こすことにより歯周組織の炎症増悪と創傷治癒の遅延をもたらす。

ている。血管内に侵入した歯周病原細菌も、末梢に伝搬され血管や末梢臓器に定着し、同部位において炎症反応を惹起する可能性が示唆されている。歯周病原細菌の多くはグラム陰性菌であり、その細胞壁成分であるリポ多糖（LPS）は強力な炎症惹起物質である。また、最も重要な歯周病原細菌の一つである *Porphyromonas gingivalis* は、強力なトリプシン様プロテアーゼ gingipain を産生し、血液凝固反応を亢進するとともに血管の炎症を惹起する（図２）。最近我々は、このgingipainが血管形成調節因子angiotoietin 2 を含む分泌顆粒のエキソサイトーシスを誘導し、LPSに対する血管内皮細胞の感受性を高め、血管の炎症応答を増強することを明らかにした[35]（図３）。歯周病原細菌由来のこれらの因子は、協調的に作用して歯周局所の炎症を増悪するとともに、全身の血管に作用して血管の

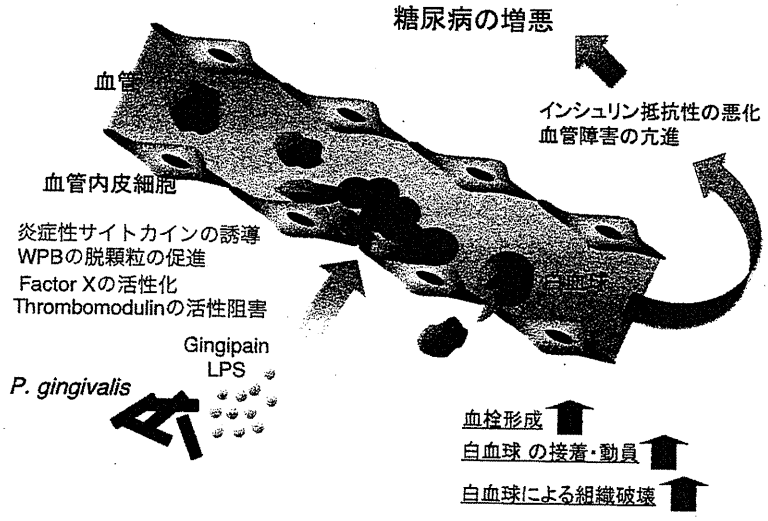

図２　血管を基盤とした歯周病による糖尿病の増悪機序

歯周病原細菌の産生するトリプシン様酵gingipainやリポ多糖は、歯周組織および全身の血管に作用し、炎症反応の増強や血栓形成を促進することにより糖尿病の病態を増悪する可能性がある。

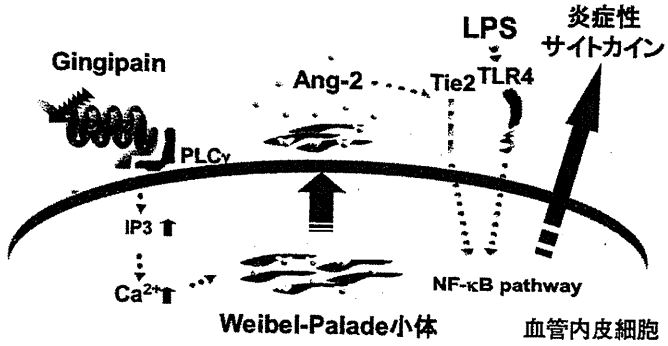

図３　歯周病原細菌由来プロテアーゼによる血管の免疫応答の増幅機序

歯周病原細菌 P. gingivalisの産生する gingipainは血管内皮のエキソサイトーシスを誘導し、LPSに対する血管内皮の免疫応答を増強する。

炎症を増強し動脈硬化を促進している可能性がある。加えて、それらの因子は糖尿病に合併する微小血管と大血管の障害を増悪する可能性が考えられる。

また、歯周病と脳卒中との相関関係も報告されていたり[36,37]、歯周病は誤嚥性肺炎のリスク要因であったりするので、高齢者においては特にそのケアが重要である[38,39]。

3）口腔乾燥症

ドライマウスとも呼ばれるが、唾液の分泌低下をきたす疾患である。加齢による唾液の分泌量が低下によって起こるというよりも、特定の薬の服用によって唾液の分泌量が減って口腔乾燥をきたすケースが多い。具体的には、降圧剤の一種であるACE阻害剤、α,βブロッカー、カルシウム拮抗剤や向精神薬等の服用は口腔乾燥の原因となる。また、シェーグレン症候群などの自己免疫疾患で口腔乾燥をきたす場合もある。病因としては、自己抗体の存在が認められる。

その基盤にはCD４＋Ｔ細胞の免疫寛容の喪失が推察されている[40]が、明確な原因は未だ不明である。Tregsの機能低下が何らかの関与をしている可能性も考えられる[41]。

4）口腔カンジダ症

Candida albicans などのカンジダ属の真菌は、健康な成人の口腔内に常在細菌叢として生息しているが、免疫能が極度に減弱した高齢者においては同菌の過剰な増殖が起こり、口腔粘膜に強度の炎症反応をきたす。唾液中には、様々な抗菌ペプチドが存在し、口腔内の常在細菌叢を制御している。中でもhistatinという抗菌ペプチドは口腔内の真菌の増殖を制御している。しかし、唾液の分泌が低下するとそれらの分泌量も減少するため、*C. albicans* の増殖を許すことになる[42]。また、老化による口腔粘膜のバリアー機能の低下は同菌の定着と侵入を容易にしている可能性がある[43]。また、粘膜に定着した*C. albicans* を排除するための細胞性免疫も低下しているために、難治化するものと考えられる。

5）口腔癌

口腔癌も高齢者において発症率の高まる疾患である。喫煙、ヒトパピローマウイルスの感染、慢性的な刺激等が危険因子に上げられるが、老化による細胞性免疫応答の低下も重要な危険因子と考えられる。NK細胞あるいは細胞傷害性T細胞等の増殖や活性の低下は、容易に癌細胞の増殖を許してしまうことになる可能性がある。

おわりに

高齢者においては、特有の口腔疾患が発症したり、また成人に発症するものとは様相が異なったりしている。その原因の一つとして、加齢による免疫能の低下が考えられる。その詳細な機序は不明な部分が多くその解明が待たれる。また、老化による免疫系の低下は避けられないことであるが、低下のスピードを緩やかにしたりすることができれば、健やかに老いることに大いに貢献できるであろう。

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Porphyromonas gingivalis is widely distributed in subgingival plaque biofilm of elderly subjects

Yuki Abiko, Takuichi Sato, Kenji Matsushita, Reiko Sakashita, and Nobuhiro Takahashi

Abstract. The frequency of periodontal diseases appears to increase with age. *Porphyromonas gingivalis* is widely regarded as major periodontal pathogens. This study aimed to quantify *P. gingivalis* in subgingival plaque biofilm of elderly subjects by real-time polymerase chain reaction (PCR). Subgingival plaque was obtained from 198 periodontally healthy (mean age, 70.3 years) and 176 subjects with periodontitis (70.6 years). Quantification of total bacteria and *P. gingivalis* was performed by real-time PCR using universal and *P. gingivalis*-specific primers based on 16S rRNA genes, respectively. Both the detection frequency and mean proportion of *P. gingivalis* were significantly higher in subjects with periodontitis than in periodontally healthy subjects ($p<0.0001$). Nevertheless, *P. gingivalis* was detected frequently both from subjects with periodontitis and periodontally healthy subjects. These results suggest that *P. gingivalis* is widely distributed in subgingival plaque biofilm of elderly subjects.

Key words. elderly subjects, plaque biofilm, *Porphyromonas gingivalis*, quantitative polymerase chain reaction, 16S ribosomal RNA

1 Introduction

Qualitative and quantitative changes of the subgingival plaque biofilm microflora in periodontal pockets are thought to be associated with the development and progression of periodontitis. The frequency of periodontal diseases appears to increase

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with age. However, few studies have been investigated on subgingival microflora in elderly subjects. *Porphyromonas gingivalis* is widely regarded as major periodontal pathogens. This study aimed to quantify *P. gingivalis* in subgingival plaque biofilm of elderly subjects by real-time polymerase chain reaction (PCR).

2 Relationship Between Periodontal Status and *P. gingivalis* in Subgingival Plaque Biofilm of Elderly Subjects

Subgingival plaque was obtained from independent elderly subjects (60 years and over, $n=374$) from gingival crevices with the deepest probing depths. Gender, probing depth, bleeding on probing (BOP) and wearing of denture were examined and recorded. Quantification of total bacteria and *P. gingivalis* was performed by real-time PCR using universal and *P. gingivalis*-specific primers based on 16S rRNA genes [1], respectively. According to the deepest probing depths, 198 (mean age, 70.3 years) were considered as periodontally healthy, while 176 (70.6 years) were considered as subjects with periodontitis. Both the detection frequency and mean proportion of *P. gingivalis* were significantly higher in subjects with periodontitis than in periodontally healthy subjects ($p<0.0001$). The proportion of *P. gingivalis* was significantly higher in subjects with BOP-positive ($p<0.05$), but not related to subjects' gender, wearing of denture and age.

3 Inhabitation of *P. gingivalis* in Subgingival Plaque of Elderly Subjects

It has been reported that *P. gingivalis* is generally detected from subjects with periodontitis [1, 2]. However, in this study, *P. gingivalis* was detected frequently both from subjects with periodontitis and periodontally healthy subjects, suggesting that the inhabitation of *P. gingivalis* is a specific feature of subgingival plaque biofilm of elderly subjects. Amano et al. [3] reported that the pathogenicity of *P. gingivalis* was different among strains, suggesting the possibility that low pathogenic *P. gingivalis* strains colonized in periodontally healthy elderly subjects in this study.

4 Clinical Implication

This study suggests that the inhabitation of *P. gingivalis* in subgingival plaque biofilm of elderly subjects is one of the risk factors of periodontitis, as well as the decrease of host defense mechanism with age, although further study is required to elucidate the pathogenicity of *P. gingivalis* found in periodontally healthy elderly

subjects. The findings of this study support the necessity and importance of periodontal control, especially in elderly subjects.

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ARTICLES

Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells

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Induced pluripotent stem cells (iPSCs) have been generated by enforced expression of defined sets of transcription factors in somatic cells. It remains controversial whether iPSCs are molecularly and functionally equivalent to blastocyst-derived embryonic stem (ES) cells. By comparing genetically identical mouse ES cells and iPSCs, we show here that their overall messenger RNA and microRNA expression patterns are indistinguishable with the exception of a few transcripts encoded within the imprinted *Dlk1–Dio3* gene cluster on chromosome 12qF1, which were aberrantly silenced in most of the iPSC clones. Consistent with a developmental role of the *Dlk1–Dio3* gene cluster, these iPSC clones contributed poorly to chimaeras and failed to support the development of entirely iPSC-derived animals ('all-iPSC mice'). In contrast, iPSC clones with normal expression of the *Dlk1–Dio3* cluster contributed to high-grade chimaeras and generated viable all-iPSC mice. Notably, treatment of an iPSC clone that had silenced *Dlk1–Dio3* with a histone deacetylase inhibitor reactivated the locus and rescued its ability to support full-term development of all-iPSC mice. Thus, the expression state of a single imprinted gene cluster seems to distinguish most murine iPSCs from ES cells and allows for the prospective identification of iPSC clones that have the full development potential of ES cells.

Induced pluripotent stem cells (iPSCs), generated by the overexpression of transcription factors such as Oct4 (also called Pou5f1), Sox2, Klf4 and c-Myc in somatic cells[1,2], have enormous therapeutic potential as they enable the derivation of patient-specific pluripotent cell lines to study and possibly treat degenerative diseases. Although the generation of iPSCs is technically simple and ethically uncontroversial, it remains unclear whether iPSCs are molecularly and functionally different from ES cells derived from blastocysts, which are considered the gold standard for pluripotent cells. Previously published reports indicate high similarities between ES cells and iPSCs, including indistinguishable global histone modification and DNA methylation patterns[3,4]. In addition, iPSCs, like ES cells, give rise to numerous differentiated cell types, including the germ line, in the context of chimaeric animals[5,6]. More recently, iPSCs have been shown to support the development of all-iPSC mice using tetraploid (4n) embryo complementation[7–9], the most stringent assay for developmental potential[10,11].

Despite these similarities, there is emerging evidence for substantial differences between ES cells and iPSCs. For example, most iPSC clones give rise to low-grade chimaeras after injection into diploid blastocysts and fail to support the development of postnatal all-iPSC mice upon 4n embryo complementation[12–14]. At the molecular level, major differences in mRNA and microRNA (miRNA) expression[15–17], as well as in DNA methylation[18–20], have been reported between ES cells and iPSCs. These observations indicate that factor-mediated reprogramming results in abnormalities in resultant iPSCs, which could impede their therapeutic utility. In contrast, nuclear-transfer-mediated reprogramming gives rise to nuclear transfer ES cells that are molecularly and functionality indistinguishable from ES cells derived from fertilized embryos[21,22], raising the possibility that nuclear transfer generates cells that are more completely reprogrammed than iPSCs.

A potential limitation of the aforementioned studies is that ES cells were compared with iPSCs of different genetic backgrounds and harbouring viral transgenes, which are known to affect gene expression patterns[21,23] and the functionality[1,6] of cells. We therefore revisited the question of whether ES cells and iPSCs are equivalent by comparing genetically matched cell lines.

Transcriptional comparison of ES cells and iPSCs

To circumvent the potentially confounding effects of genetic background and viral integrations on gene expression patterns and developmental potential, we used a novel transgenic reprogramming system to generate genetically matched mouse ES cells and iPSCs[24]. Briefly, a polycistronic cassette expressing Oct4, Klf4, Sox2 and c-Myc[25] (OKSM) under the control of a doxycycline-inducible promoter was inserted into the collagen type I α1 (*Col1a1*) locus of ES cells expressing the reverse tetracycline-dependent transactivator (rtTA) from the *ROSA26* promoter[26]. These collagen-OKSM ES cells were then used to generate mice from which different somatic cell types were isolated and induced with doxycycline to derive genetically matched iPSCs for molecular and functional comparisons (Fig. 1a, b).

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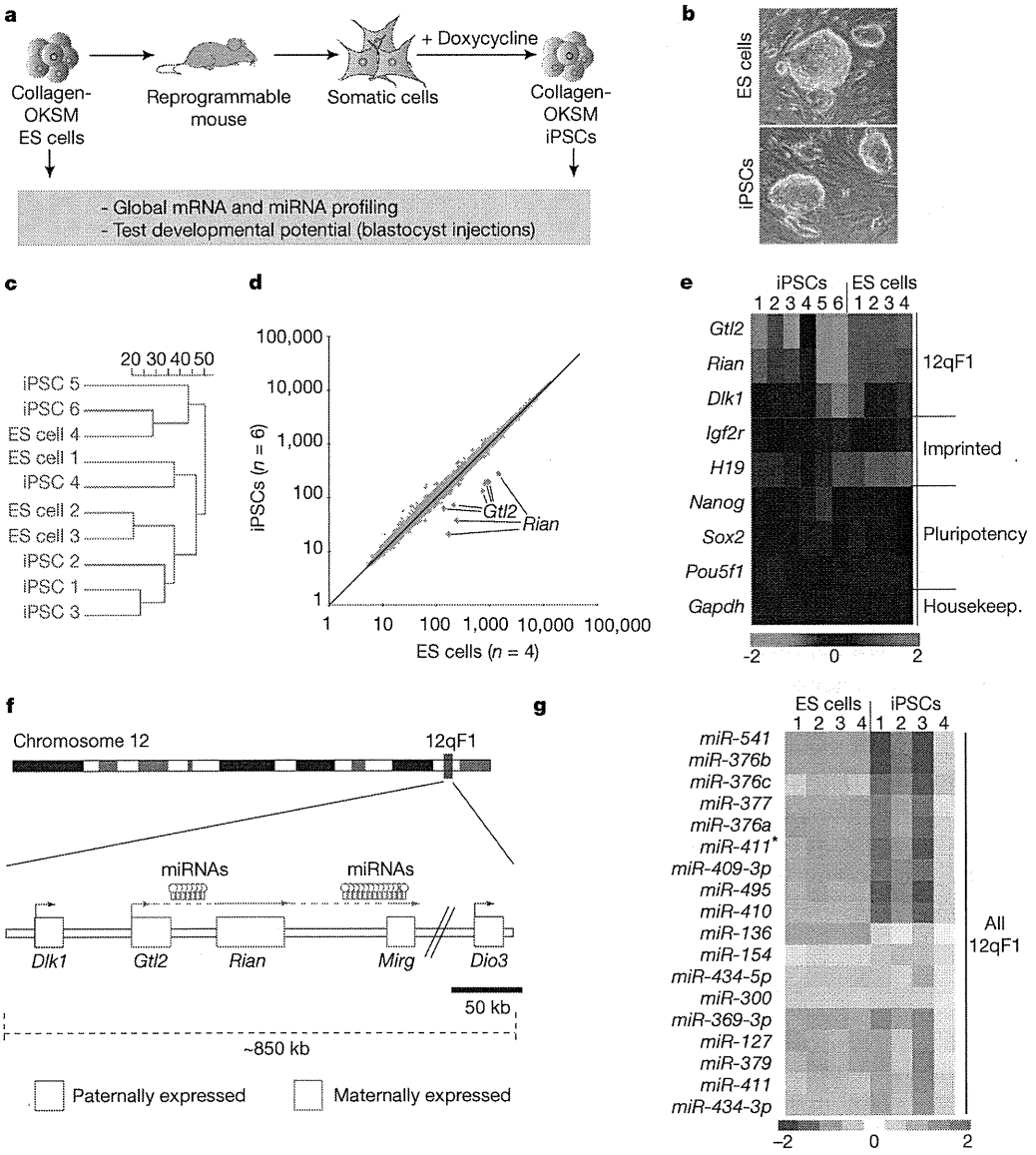

Figure 1 | Aberrant silencing of the *Dlk1–Dio3* gene cluster in mouse iPSCs. **a**, Strategy for comparing genetically matched ES cells and iPSCs generated with the doxycycline-controllable collagen-OKSM system. **b**, Morphology of collagen-OKSM ES cells and iPSCs. **c**, Unsupervised clustering of four ES cell and six derivative iPSC lines based on microarray expression data. **d**, Scatter plot of microarray data comparing iPSCs and ES cells with differentially expressed genes highlighted in green (twofold, $P < 0.05$, *t*-test with Benjamini–Hochberg correction). **e**, Heat map showing relative expression levels of selected mRNAs in ES cells and iPSCs. **f**, Schematic representation of the *Dlk1–Dio3* gene cluster with maternally and paternally expressed transcripts shown in red and blue, respectively. **g**, Heat map showing miRNAs that are differentially expressed between ES cells and iPSCs (twofold, $P < 0.01$, *t*-test).

We first compared the abilities of parental collagen-OKSM ES cells and iPSCs derived from mouse embryonic fibroblasts (MEFs) to support the development of all-iPSC mice using 4n embryo complementation. The two tested ES cell lines gave rise to viable mice at expected frequencies (13–20%)[11], demonstrating that the OKSM transgene per se does not adversely affect development (Supplementary Table 1). In contrast, all four tested iPSC lines repeatedly failed to support the development of all-iPSC mice, indicating qualitative differences between these iPSCs and ES cells (Supplementary Table 1).

We reasoned that a transcriptional comparison of the iPSC lines that failed 4n complementation with matched 4n-complementation-competent ES cell lines might reveal molecular changes that explain the developmental deficits of iPSCs. Global mRNA profiling showed marked similarities in the overall transcriptional patterns of four collagen-OKSM ES cells and six derivative iPSCs and did not separate these cell lines using unsupervised clustering or principal component analysis (Fig. 1c and data not shown). In fact, only two transcripts were identified as differentially expressed (>2-fold difference, *t*-test, $P < 0.05$) between ES cells and iPSCs. These were the non-coding RNA *Gtl2* (also know as *Meg3*) and the small nucleolar RNA *Rian* (Fig. 1d, e).

Repression of *Dlk1–Dio3* transcripts in iPSCs

Gtl2 and *Rian* localize to the imprinted *Dlk1–Dio3* gene cluster on mouse chromosome 12qF1 and are maternally expressed in mammals (Fig. 1f)[27]. Both genes were strongly repressed in iPSC clones compared to ES cell clones, whereas expression of pluripotency and housekeeping genes remained unaffected (Fig. 1e). Quantitative PCR (qPCR) analysis of *Gtl2*, *Rian* and *Mirg*, another maternally expressed imprinted gene in the *Dlk1–Dio3* cluster, confirmed transcriptional silencing in iPSCs (Supplementary Fig. 1a). Expression of other imprinted genes showed clone-to-clone variations, as was previously seen for ES cells[28], but no consistent differences between ES cells and iPSCs (Fig. 1e and Supplementary Table 2). This shows that imprinted gene silencing is not a genome-wide phenomenon. Of note, we failed to detect differences with the collagen-OKSM system

in the expression of ~300 transcripts that have recently been reported to be differentially regulated in a comparison of mouse and human iPSCs with ES cells[15] (Supplementary Fig. 2a). However, silencing of *Gtl2* was also evident in the murine-specific iPSC data set used in that study (Supplementary Fig. 2c). Together, these data indicate that a relatively small set of transcripts distinguishes genetically matched iPSCs and ES cells, and suggest that many of the previously seen differences are probably due to variations in genetic background or viral transgene insertions.

Imprinting of the *Dlk1–Dio3* locus is accompanied by differential expression of about 50 miRNAs (Fig. 1f)[29,30]. We therefore performed genome-wide miRNA profiling on the same samples as analysed for mRNA expression. Of 336 miRNAs detected, 21 (6.3%) were differentially expressed between all ES cell and iPSC clones analysed (Fig. 1g and Supplementary Table 3). All of these miRNAs localized to chromosome 12qF1 and were silenced in iPSCs, thus corroborating the notion that most iPSCs show aberrant silencing of this major imprinting domain.

To determine the generality of *Gtl2* silencing in iPSCs, we measured its expression in 61 additional iPSC lines derived from haematopoietic stem cells (11 lines), granulocyte-macrophage progenitors (11 lines), granulocytes (9 lines), peritoneal fibroblasts (6 lines), tail-tip fibroblasts (6 lines) and keratinocytes (18 lines). Only four of these lines (5.8%), originating from either peritoneal or tail-tip fibroblasts, showed *Gtl2* expression levels similar to those of ES cells (termed 'Gtl2^{on} clones') (Fig. 2a and Supplementary Fig. 1b, c). The finding that the vast majority of iPSC clones showed transcriptional suppression of *Gtl2* (termed 'Gtl2^{off} clones') demonstrates that silencing of this locus occurs in iPSCs derived from different cell types at various stages of differentiation. Analysis of published microarray data sets comparing ES cells and iPSCs generated from mouse fibroblasts, neural and bone marrow cells also showed repression of maternally expressed 12qF1 transcripts (Supplementary Fig. 2b–e), supporting the notion that silencing of this cluster is a common outcome upon factor-mediated reprogramming.

Developmental effects of *Dlk1–Dio3* silencing

Dysregulation of genes within the *Dlk1–Dio3* cluster can be detrimental during pre- and postnatal mouse development[31–35]. To assess whether the expression status of *Gtl2* and its associated transcripts

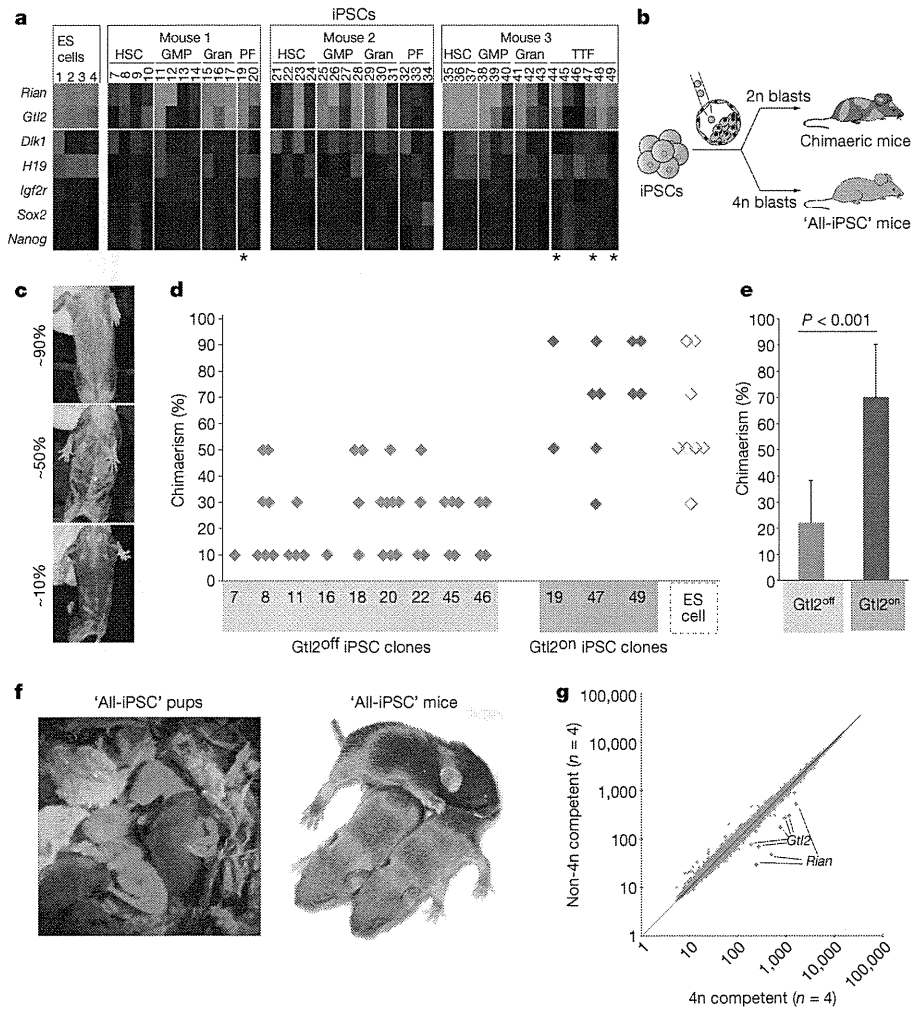

Figure 2 | Developmental consequences of *Dlk1–Dio3* silencing. **a**, Heat map showing relative expression levels of *Gtl2*, *Rian* and other select genes in ES cells and iPSCs derived from haematopoietic stem cells (HSC), granulocyte-macrophage progenitors (GMP), granulocytes (Gran), peritoneal fibroblasts (PF) and tail-tip fibroblasts (TTF). Four iPSC clones expressing ES-cell-like levels of *Gtl2* and *Rian* were identified (highlighted by asterisks) (iPSC clone number 18 was analysed only by qPCR; see Supplementary Fig. 1b). **b**, Strategy for assessing the developmental potential of iPSC clones by injection into diploid (2n) and tetraploid (4n) blastocysts to produce chimaeric or all-iPSC mice, respectively. **c**, Images of representative chimaeras with agouti coat colour indicating iPSC origin. **d**, Quantification of coat colour chimaerism in mice derived from indicated Gtl2^{off} clones (green diamonds), Gtl2^{on} iPSC clones (red diamonds) and ES cells (open diamonds). **e**, Statistical analysis of coat colour chimaerism in mice derived form Gtl2^{off} and Gtl2^{on} iPSC clones. Error bars indicate standard deviations, $n = 38$ for Gtl2^{off} clones and $n = 11$ for Gtl2^{on} clones, $P < 0.001$. **f**, Images of two GFP^{+} all-iPSC neonates (left) and two agouti all-iPSC mice (right). **g**, Scatter plot showing intensity levels of all probe sets covered by microarray analysis; highlighted in green are those probe sets that were significantly different between 4n complementation-competent iPSCs (clones 19, 44, 47 and 49) and non-4n complementation-competent iPSCs (clones 18, 20, 45 and 48) (twofold, $P < 0.05$, *t*-test with Benjamini–Hochberg correction).

correlates with the developmental potential of iPSCs, we injected nine Gtl2^{off} clones into diploid blastocysts. This resulted in adult chimaeras that exhibited low-to-medium degree (10–50%) coat colour chimaerism (Fig. 2b–e and Supplementary Table 4). In contrast, injection of three Gtl2^{on} iPSC clones yielded adult mice with a coat colour chimaerism ranging from 30% to 100%, similar to the chimaerism seen with ES cells (Fig. 2d, e and Supplementary Table 4). Importantly, four out of four Gtl2^{on} iPSC clones supported the development of neonatal all-iPSC mice upon injection into 4n blastocysts at efficiencies comparable to those observed with ES cells (7–19% for iPSCs compared with 13–20% for ES cells) (Supplementary Table 1). We confirmed that these mice were entirely iPSC-derived by PCR for strain-specific polymorphisms (Supplementary Fig. 3), by detection of homogenous GFP fluorescence of all-iPSC neonates, originating from a *ROSA26-EGFP* allele introduced into the parental ES cells, and by uniform agouti coat colour of adolescent all-iPSC mice (Fig. 2f). To our knowledge, this is the first demonstration of animals produced entirely from adult-derived iPSCs.

In contrast to Gtl2^{on} iPSC clones, injection of ten Gtl2^{off} iPSC clones into 4n blastocysts consistently failed to produce all-iPSC pups but instead resulted in resorptions (Supplementary Table 1). Thus, the expression status of *Gtl2* in these iPSCs predicts their developmental potential into chimaeric and all-iPSC mice. The conclusion that the activation status of maternally expressed genes on chromosome 12qF1 is a strong indicator of the developmental potential of iPSCs was further supported by the analysis of two published array data sets, which showed that *Gtl2* was expressed in 4n complementation-competent ES cell and iPSC lines but was downregulated in non-4n complementation-competent iPSC lines[8,9] (Supplementary Fig. 4).

To test whether Gtl2^{on} and Gtl2^{off} iPSCs can be distinguished by the expression of genes outside of 12qF1, we performed global mRNA and miRNA expression profiling of four non-4n complementation-competent and four 4n complementation-competent iPSC lines (all derived from fibroblasts). This analysis identified only *Gtl2*, *Rian* and a total of 26 miRNAs, which all localize to the *Dlk1–Dio3* cluster, as differentially expressed, suggesting that the dysregulation of 12qF1 transcripts alone is responsible for the failure of Gtl2^{off} iPSCs to support the development of all-iPSC mice (Fig. 2g and Supplementary Table 5).

Epigenetic mechanism of *Dlk1–Dio3* repression

Imprinting of the *Dlk1–Dio3* cluster is regulated by differentially methylated regions (DMRs) that become epigenetically modified in the germ line. These include an intergenic DMR (IG-DMR), located between the *Dlk1* and *Glt2* genes[19], and a DMR spanning the *Gtl2* promoter (*Gtl2* DMR)[15]. To determine whether aberrant DNA methylation might be responsible for the transcriptional silencing seen in Gtl2^{off} iPSC lines, we compared the methylation status of IG-DMR, *Gtl2* DMR and three other CpG-rich regions on chromosome 12qF1 in ES cells, iPSCs and their parental tail-tip fibroblasts (Fig. 3a). As expected for germline-imprinted regions,

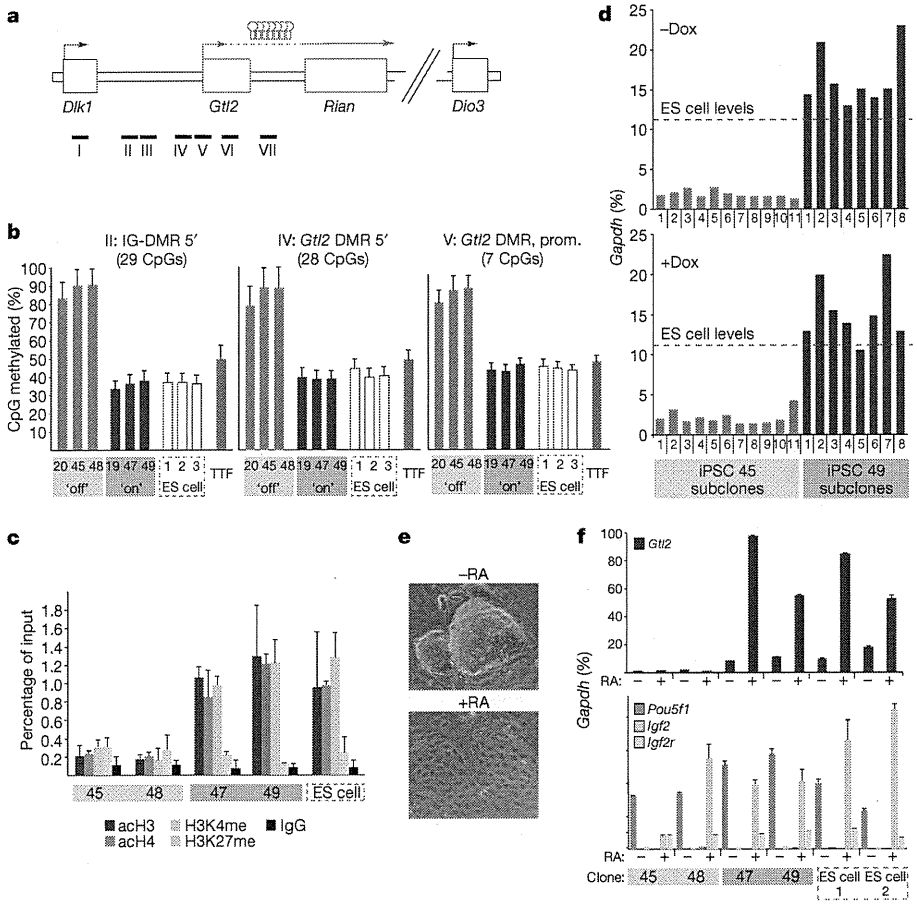

Figure 3 | Epigenetic silencing of the *Gtl2* locus in iPSCs. a, Structure of the *Dlk1–Dio3* locus with the position of the genomic regions (I–VII) analysed by pyrosequencing indicated by black bars. **b**, Degree of DNA methylation at IG-DMR and *Gtl2* DMR in three Gtl2^{off} iPSC clones (green bars), three Gtl2^{on} iPSC clones (red bars), three ES cell clones (red open bars), as well as the parental tail-tip fibroblasts (TTF, grey bars). Analysis of the other regions is shown in Supplementary Fig. 5. **c**, Prevalence of activation-associated (acH3, acH4 and H3K4me) and repression-associated (H3K27me) chromatin marks at the *Gtl2* promoter in two Gtl2^{off} iPSC clones, two Gtl2^{on} iPSC clones and ES cells. **d**, *Gtl2* expression levels as measured by qPCR in subclones derived from Gtl2^{off} clone 45 and Gtl2^{on} clone 49 in the absence (−) or presence (+) of doxycycline (Dox). **e**, Bright-field images of iPSC culture in the absence or presence of all-trans retinoic acid (RA). **f**, Expression levels of *Gtl2*, other imprinted genes (*Igf2*, *Igf2r*), and the pluripotency marker *Pou5f1* in cells cultured with (+) or without (−) RA. All error bars indicate standard deviations with n = number of CpGs within the corresponding region in **b** and $n = 3$ in **c** and **f**.

approximately 50% of CpGs within IG-DMR and *Gtl2* DMR were methylated in fibroblasts, ES cells and Gtl2^{on} iPSCs. In contrast, close to 100% of these CpGs were methylated in Gtl2^{off} iPSC lines (Fig. 3b and Supplementary Fig. 5). The other analysed CpG-rich regions remained unaffected (Supplementary Fig. 5). Imprinting of the *Dlk1–Dio3* cluster is also regulated by histone acetylation[36], and chromatin immunoprecipitation experiments indeed revealed a significant decrease in acetylated H3 and H4 as well as in methylated H3K4, another activation mark, in Gtl2^{off} iPSC lines compared with Gtl2^{on} iPSC lines and ES cells (Fig. 3c). Together, these observations demonstrate that the normally expressed maternal *Gtl2* allele has acquired an aberrant paternal-like silent state in Gtl2^{off} iPSC clones.

Because imprinted gene expression is unstable in murine ES cells[28,37], we assessed *Gtl2* expression upon subcloning of iPSCs. *Gtl2* remained silent in subclones from Gtl2^{off} iPSCs and continued to be expressed in subclones from Gtl2^{on} iPSCs, demonstrating stability of its expression state in undifferentiated cells (Fig. 3d, top). This pattern was not altered if doxycycline was administered during the subcloning procedure (Fig. 3d, bottom), indicating that overexpression of the reprogramming factors in established iPSCs is insufficient to induce or revert silencing.

To assess if silencing of *Gtl2* is resolved during differentiation, we exposed iPSCs and ES cells to the differentiation-stimulating agent retinoic acid. Marked changes in cellular morphology and downregulation of *Pou5f1* indicated successful differentiation (Fig. 3e, f). Whereas retinoic-acid-treated Gtl2^{on} iPSCs and ES cells readily upregulated *Gtl2* (Fig. 3f, top) and *Rian* (Supplementary Fig. 6), Gtl2^{off} iPSCs showed stable silencing of these genes, demonstrating that *in vitro* differentiation fails to reactivate maternally imprinted genes in the *Dlk1–Dio3* cluster. The expression of imprinted genes outside of chromosome 12qF1 was not affected (Fig. 3f, bottom, and Supplementary Fig. 6).

We next sought to gain an insight into the causes for the failure of Gtl2^{off} iPSCs to produce viable all-iPSC mice. To this end, we determined if these cells could autonomously support development into early embryos. Indeed, injection of both Gtl2^{off} and Gtl2^{on} iPSC clones into 4n blastocysts gave rise to normal-appearing mid-gestation (embryonic day (E) 11.5) embryos (Fig. 4a). However, the number of live embryos obtained from Gtl2^{off} clones was substantially reduced compared with Gtl2^{on} clones (Fig. 4b), suggesting that Gtl2^{off} mice die around this developmental stage. This phenotype resembles that of mice with paternal uniparental disomy of distal chromosome 12[38], which die before E16.5, but is distinct from the phenotype of maternal *Gtl2* knockout mice (Gtl2^{mKO}), which die perinatally[31]. The less severe phenotype of Gtl2^{mKO} embryos might be due to the comparably modest reduction in maternally expressed 12qF1 genes[31]. In agreement with this notion, we found low but detectable levels of *Rian* and *Mirg* transcripts in Gtl2^{mKO} MEFs (Fig. 4c), whereas these genes were almost completely silenced in MEFs and different tissues derived from Gtl2^{off} all-iPSC embryos (Fig. 4d, e).

Notably, expression of the *Dlk1* gene, which is reciprocally imprinted to *Gtl2* (ref. 39), was upregulated in Gtl2^{off} MEFs but not in Gtl2^{mKO} MEFs (Fig. 4c), further supporting the observation

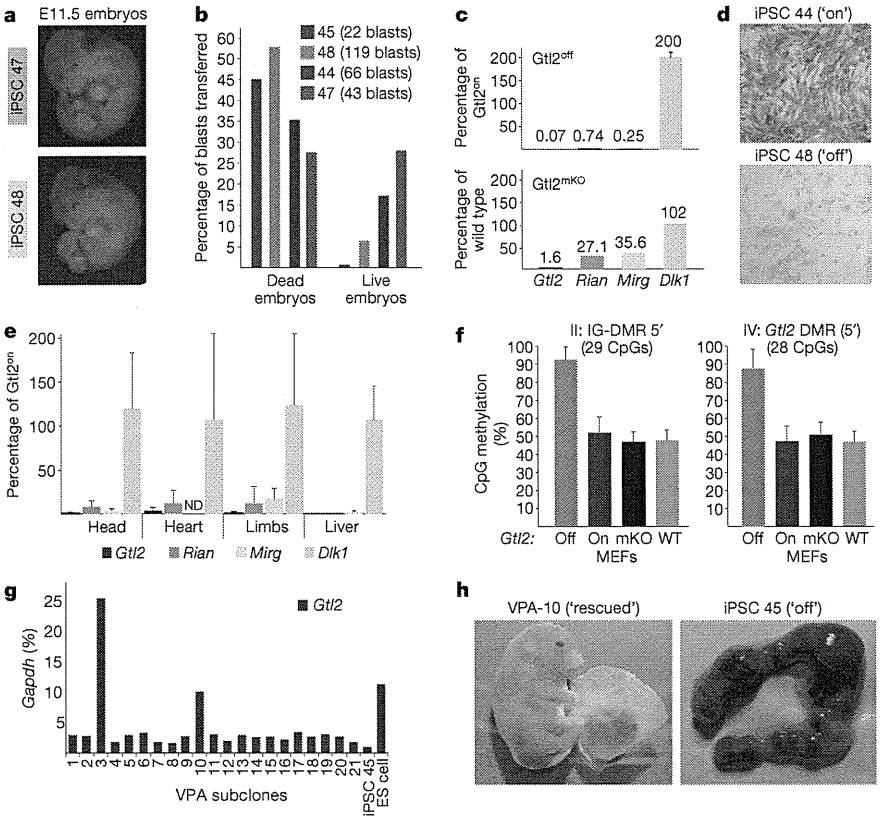

Figure 4 | Developmental defects in embryos derived from Gtl2^{off} iPSCs. **a**, Images of 'all-iPSC' E11.5 embryos obtained with Gtl2^{on} clone 47 and Gtl2^{off} clone 48, both of which express EGFP ubiquitously from the *ROSA26* locus. **b**, Frequency of dead and live E11.5 all-iPSC embryos obtained with two Gtl2^{on} (red bars) and two Gtl2^{off} (green bars) iPSC clones upon 4n blastocyst injection. Number of blastocysts transferred per clone is indicated in brackets. **c**, Expression of *Gtl2*, *Rian*, *Mirg* and the paternally expressed gene *Dlk1* in Gtl2^{off} MEFs relative to Gtl2^{on} MEFs (upper panel) as well as in Gtl2^{mKO} MEFs relative to MEFs isolated from wild-type embryos (lower panel). **d**, *In situ* hybridization for *Gtl2* mRNA in MEFs derived from all-iPSC embryos generated with either Gtl2^{on} clone 44 or Gtl2^{off} clone 48. **e**, Expression levels of *Gtl2*, *Rian*, *Mirg* and *Dlk1* in the indicated tissues isolated from all-iPSC embryos produced with Gtl2^{off} iPSCs relative to the levels seen in tissues derived from Gtl2^{on} iPSCs. **f**, Degree of DNA methylation at the indicated *Dlk1–Dio3* regions in Gtl2^{off}, Gtl2^{on}, Gtl2^{mKO} and wild-type MEFs. **g**, *Gtl2* expression levels in iPSC lines derived by subcloning Gtl2^{off} clone 45 in the presence of valproic acid (VPA). **h**, Images of a neonatal stillborn pup (left) and a uterus filled with resorptions (right) derived after 4n blastocyst injections with either VPA-10 or the parental iPSC clone 45, respectively. All error bars indicate standard deviations with $n = 3$ in **c**, $n = 5$ in **e** and n = number of CpGs in **f**.