ムノクロマトを用いた抗SARS抗体(IgG)を検出するキットが入手可能になってきた。

Ⅱ SARS臨床検査の原則

WHOは2003年5月に「臨床検査結果の解釈に関する提言」及び「SARSの臨床検査に対する提言」を公開している[2)]。その中で「国レベルでリファレンス・ラボを指定しなければならない」と提言している。日本では，感染研がこれにあたり，地衛研と共同で精度管理を実施している[1)]。また，SARS診断検査の陽性として，以下のように定義している。

a）SARSコロナウイルスPCR陽性
- 最低2つの異なる臨床検体（たとえば，鼻咽頭拭いと糞便）で陽性である。
- 同一の臨床検体が臨床経過中に2日以上に渡って採取された検体で陽性である(たとえば，2つ以上の鼻咽頭吸引検体)。
- 2つの異なる検査法，または検査を反復して実施しても陽性である。

b）ELISAあるいはIFAで抗体陽性
- 急性期血清において抗体陰性で，続いて回復期血清において抗体陽性である。
- 急性期と回復期の血清が平行して検査され，4倍以上の抗体価の上昇を回復期血清で認める。

c）ウイルス分離陽性
- PCR法で陽性が確認された検体で，ウイルス培養によってSARSコロナウイルスが分離された場合。

PCR陽性の確認として，検査一回ごとに適切な陰性と陽性のコントロール(対照検体)をとる必要がある。具体的には，以下のものである。
- 抽出過程に陰性検体と，PCR増幅過程に水検体を陰性コントロールとしておく。
- PCR増幅過程と抽出過程に陽性検体をそれぞれおく。
- PCR検査を阻害する物質を検出するため，弱陽性のコントロールを患者検体に混ぜたものを検査する（インヒビション・コントロール)。

もしPCR結果が陽性であった場合には，結果の確認を次のような方法で行う。
- 原検体から反復してPCRを行う。

または，
- 他施設で同一の検体を検査する。

日本におけるSARSのリファレンス・ラボは，感染研である。ここではSARSコロナウイルスに関する特異的検査を行うが，それ以外の既知の病原体の検査は地衛研もしくは病院検査部で行う。疑い例と可能性例の全員についてSARSコロナウイルス特異的ウイルス検査を実施する。疑い例でSARSコロナウイルス特異的検査において，複数の施設の結果が陽性であった場合，その時点で可能性例として扱う。SARSコロナウイルス以外の病原体で，既知の肺炎を起こす異型肺炎含む病原体の一次スクリーニングを行う。スクリーニングとして，一般細菌培養，迅速診断法（連鎖球菌など一般細菌，レジオネラ，クラミジア，マイコプラズマ，アデノウイルス，インフルエンザウイルス，RSウイルス，その他について，地域における患者発生状況を考慮して，必要な病原体について検討する)，血清学的方法（マイコプラズマ，クラミジア）を含んでいる。

感染研（ウイルス第三部）のSARSコロナウイルスの検査対応が公開されているので図1に示す。図にあるように遺伝子診断とウイルス分離は対応できるが，現時点では抗体検査は陽性となる抗血清がなく実施できていない。SARSコロナウイルスの場合，電子顕微鏡も有力な診断法になる。

Ⅲ SARSの臨床検査上の特性

SARSコロナウイルス感染は，臨床ウイルス学上以下の3点が大きな特徴となっている。まず，SARS感染の初期には，感染者からのウイルスの排出が少なく，ウイルスの排出のピークは，臨床症状の出現後10日目前後である（図2)。インフルエンザなどの多くのウイルス性疾患においては，発病初期の発症後の数日間に最も多量の原因ウイルスが排出される。この時期がしばしば，他者を感染させるリスクが最も高い期間となる。このようなSARSの特性は，院内感染などのSARS特有の伝播様式を理解するのに役に立つばかりでなく，SARS診断検査法にはより高い感受性が必要である理由となっている。2番目は，SARSコ

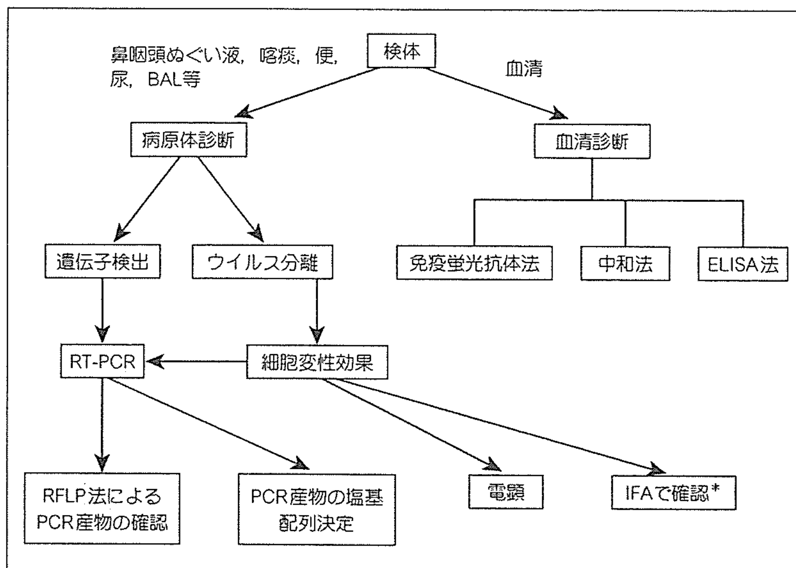


図1　SARS コロナウイルスの検査対応

SARS 診断のための検査は，病原体検査と血清検査を実施する。病原体検査ではRT-PCR 法などの遺伝子検査と行なうと共に，ウイルス分離を試みる。血清診断は免疫蛍光抗体法と ELISA 法を実施する。必要に応じて，ウイルス中和活性を観察する。
*現時点では，SARS コロナウイルス同定用の抗血清がないため，基本的には RT-PCR 法により同定検査を行う。

（国立感染症研究所ウイルス第三部が公開している図を一部改変）

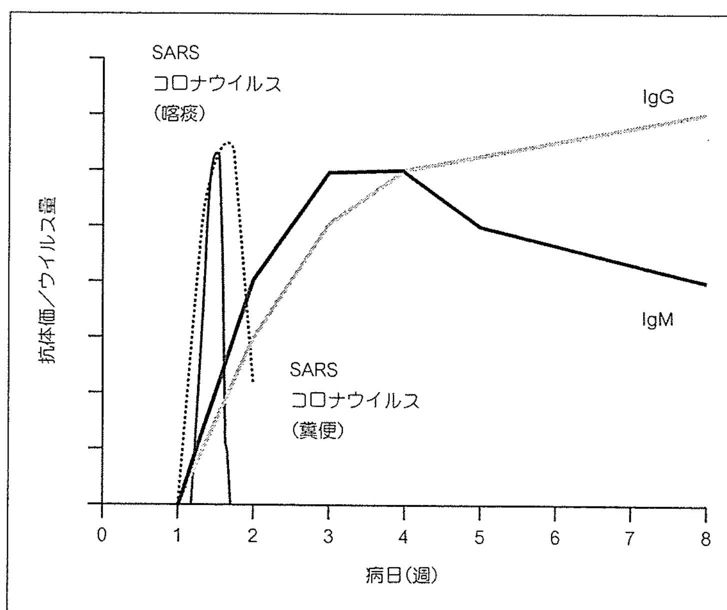


図2　排出される SARS コロナウイル量と SARS ウイルス抗原に対する抗体価の変化

典型的なSARS患者では，発病後10日目をピークに喀痰でウイルスが検出できる。15日目以降，ウイルス量は著しく減少する。糞便では喀痰より長期間ウイルスが検出される。抗体価10日目以降検出可能となる。

ロナウイルスは，喀痰などの気道分泌液ばかりでなく，血液や糞便から検出される特性を持っている。実際，糞便からの比較的多量のウイルスが長期間排出される。香港では，14病日でも97%の患者の便からウイルスが検出されている。もともと，コロナウイルスはヒトではかぜの原因として知られているが，動物では消化器疾患と呼吸器疾患を引き起こすことが知られている。例えば，豚コロナウイルスグループは，豚に消化器疾患を引き起こす2種類のウイルスと呼吸器疾患を引き起こす1種類のウイルスが知られている。ウシでは，ウシコロナウイルス（BcoV）が知られS遺伝子上のひとつの遺伝子変異によって，下痢症を発症するウイルス（BcoV-E）と呼吸器症状を引き起こすウイルス（BcoV-R）が知られている。このほか，イヌ，ネコ，マウス，ニワトリや七面鳥などに感染するコロナウイルスが知られている。SARSコロナウイルスは気道分泌液ばかりでなく，糞便から多く排出される特性を持っている。むしろ，糞便からのほうが長期にわたりウイルス遺伝子が検出できたとする報告がある。3番目の臨床検査上の特徴または注意として，SARSコロナウイルスが環境中で非常に抵抗性であることをあげる[3]。WHO研究施設ネットワークの実験データでは，糞便（尿）中では，室温で最低1～2日間ウイルスは安定である（pHが正常便中より高い）。下痢症状の患者の便中では，ウイルスは(最長4日間まで）より安定である。細胞培養上清中でのウイルスは4℃では21日後でもウイルスの感染性はほんのわずかしか減少しない。また，室温（一定）では2日間経っても，ウイルス量で10分の1までしか減少しない。ただし，一般に用いられている様々な消毒剤や家庭用洗剤や固定剤による処理で，ウイルス感染性はなくなる。

Ⅳ ウイルス分離

SARSコロナウイルスの分離は，実験室内感染を防ぐため，バイオセーフティレベル(BSL) 3の設備が必要である。シンガポールのSARSコロナウイルスを扱う研究所で実際に実験室・検査室感染が起きてしまったが，発生しないように万全の感染対策をとる。ウイルスの宿主となるVeroE6細胞，細胞培養用の培地，インキュベーターが必要である。細胞変性効果(cytopathic effect;CPE)などの分離ウィルスを同定する技術も必要である。我が国では，感染研と多くの地衛研とSARSウイルス分離の準備ができている。CPEが出現した場合には培養上清，培養細胞，当該臨床検体を感染研に送付する。地衛研においてウイルス分離が困難である場合は，臨床検体を感染研に送付する。SARSコロナウイルス検出のためのウイルス分離用検体の採取・処理法およびウイルス分離に関する詳細は，感染研のホームページで公表されている[4]。ここでは，原則を記載する。ウイルス分離検査において検体の採取日，個人差等で陰性となることがあるので，陰性の検査結果はSARSコロナウイルス感染を否定するものではない。SARSコロナウイルスはBSL3で扱う。SARS関連の患者検体の処理は，BSL 2またはそれ以上のレベルで行わなければならない。細胞への検体の接種は，BSL3で対応しなければならない。従って，必ず安全キャビネット内ですべて処理できるように，また検体の飛散や汚染の拡大が起こらないように工夫する必要がある。臨床検体からウイルスの分離率を高めるためには，採取された検体は可能な限り早期に細胞に接種される必要がある。検体採取から細胞に接種するまでは，4℃に冷やしておく。

VeroE6細胞によるウイルス分離では，SARSコロナウイルスだけでなく単純ヘルペスウイルス，エンテロウイルス，パラインフルエンザウイルス，RSウイルス，レオウイルス1，2，3，等の種々のウイルスが分離される可能性がある。このため，CPEが出現してもコロナウイルスか否かを同定する必要がある。現時点では，SARSコロナウイルス同定用の抗血清が無いため，基本的にはRT-PCR法により同定検査を行う。CPE陽性の培養上清を変性剤含有バッファー（RLT buffer）で処理するまでは，BSL3で行う。それ以降のRNA抽出操作はBSL2で行ってもよい。CPE陽性の培養上清は，分離ウイルスの同定確認試験のために，もとの臨床検体と他の病原体鑑別試験の成績とともに，感染研に送付する。CPE陽性が出た場合の最終判定は感染研究所での確認試験と合

わせて実施する。

V 遺伝子診断

1. RT-PCR法

SARSコロナウイルスは，RNAウイルスである。従って，ウイルス遺伝子を増幅同定するためには，RT-PCR法を行なわなければならない。RT-PCR法でSARSコロナウイルス遺伝子を検出するための約10種類のプライマーセットが公表されている。感染研においても，これらプライマーを用いて至適条件を検討しているが，陽性の臨床検体が限られていることから，現在のところ臨床材料から高感度にウイルス遺伝子を検出できる条件を決定するには至っていない。感染研では，現在4種類のプライマーを用いてRT-PCR法を実施している。プライマー配列や検体の処理方法などプロトコールの詳細は，感染研のホームページから公開されている[5]。PCRの感度は使用する酵素のメーカーやPCRチューブの材質，サーマルサイクラーの機種によっても大きく影響を受けることが知られている。現在まで感染研が行った検討においてはNPconS2/As1（公開されている感染研の情報を参考)のプライマーセットを用いると，比較的高感度にウイルス遺伝子を検出できると考えているが，米国，香港，フランクフルト等の研究施設からSARSコロナウイルスの全塩基配列が報告されていることから，今後さらに検出感度の高いプライマーの設計，RT - PCR条件などが改良されていくであろう。

これまでのRT-PCR法では偽陰性となることがある。従って，陰性の検査結果はSARSコロナウイルス感染を否定するものではない。また，PCR陽性検体が確認された場合，直ちに陽性と判断してはならない。WHOは，異なる検査施設においてダブルチェックを行うことを勧告している。従って，最初の臨床検体を感染研に送付し，確認試験を行った上で最終的に判定する必要がある。1検体での判定では信頼性が低い。PCRの結果の解釈に関しては，ウイルス分離の結果やペア血清における抗体価の測定結果と比較して総合的に判断する。

2. その他の遺伝子診断法の開発

現時点では前項で述べた通常のRT-PCR法が遺伝子診断の基本となるが，種々の目的のための遺伝子診断法が開発されている。

1）リアルタイムPCR法

SARSコロナウイルスに特異的なリアルタイムPCR診断キット（ロッシュ）が開発された。検出感度は1反応当たり20コピーで，他の病原細菌や病原ウイルスとの交差反応もなく特異性が高いとされている。ウイルスの定量化に優れているが，測定には特別の機器が必要である。

2）LAMP法

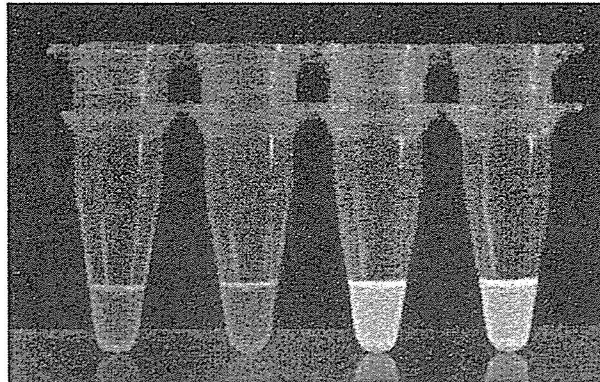
Loop-mediated Isothermal Amplificationの略で，2本鎖DNA，6つの領域を認識する4つのプライマー，鎖置換型DNA polymerase，基質等を同一容器に入れ，一定温度(65℃付近)下で，増幅から検出までを1ステップで行うことができる。増幅効率が高く，DNAを15分～1時間程度で10^9～10^{10}倍に増幅することができ，適当なプライマーを設計することにより，はじめの遺伝子増幅で両端がループ上の遺伝子産物が生成される。これをもとに遺伝子増幅を繰り返すことで，このループ構造をもったいろいろな大きさの特異的な遺伝子産物が作られる。遺伝子産物は蛍光インターカレート剤(エチジウムブロマイド)で可視化できる。この方法を用いて，感染研，長崎大学と栄研化学の共同研究でSARSウイルス用の検出キットが開発された。このキットは，反応がワンステップで，肉眼で判定可能で(図3)，感度も1反応あたり10コピーと高感度であり，迅速(20分以内)である。一定温度（65℃付近）で保温できる簡単な装置があれば，どこでも使用可能であり，検疫施設などの1次スクリーニングにもきわめて有効であると考えられる。しかし，臨床応用例が少ないため，特異性などは今後の検討が必要である。

3）その他

感染研の佐多らは，in situハイブリダイゼーション法を用いて，組織切片上でのSARSウイルスを同定した。この方法によって，病理学的観点からのSARSの病態解析が可能となってきた。

Multiplex RT-PCR法とLuminexビーズア

SARS RT-LAMPでの目視判定



SARSコロナウイルス遺伝子　−　−　＋　＋

図3　LAMP法によるSARSコロナウイルス遺伝子の同定
LAMP法では一定温度による反応でSARSウイルス遺伝子が増幅できる。増幅した遺伝子は，エチジウムブロマイドなどで簡単に可視化できる。
(納富継宣博士〔栄研生化学研究所〕のご好意で写真を提供していただいた)

レー法を用いて，SARSやインフルエンザなどの呼吸器疾患の原因となる微生物10種類を鑑別診断するキットが開発された（Genaco Biomedical Products)。この方法では，Multiplex RT-PCR法で原因微生物の遺伝子の特異領域を増幅する。このPCR産物とあらかじめLuminexビーズ上に固定化した特異的オリゴヌクレオチとをハイブリダイゼーションさせ，増幅産物と特異的に結合したビーズを同定する。Luminexビーズは，2色の蛍光物質の混合比率を変えて着色されている。ビーズの色によってビーズに固定された解析対象物を識別可能なため，1度に100種類の解析ができる。このような多くの病原微生物を一度に鑑別診断する遺伝子診断法は，微生物診断の主流になってくるであろうが，現時点では研究のための使用に限られている。

SARSゲノム情報を基づいたDNAチップも開発されている(CombiMatrix)。これは直接診断法には使用できないが，SARSコロナウイルスの疫学，より簡便な診断法開発，治療薬開発などの研究に応用されるだろう。

Ⅵ　抗体反応を用いた検査

SARS患者では，検出可能な強さの免疫反応は発病後5，6日経過しないと起こらない。信頼できる抗体検査は，発症後10日目前後になって初めて検出可能である。従って，抗体検出は，SARSの早期診断には実用的でない。しかし，SARS遺伝子診断が比較的短い期間しか陽性にならないのに比べて，抗体反応は長期間持続し，確定診断には欠かせない検査法である。抗体検査として，IFA（免疫蛍光抗体法）とELISAがあり，どちらも中国や香港などでは，確定診断として利用されてきた。しかし，陽性抗血清の入手が難しく，診断キットとしては開発が遅れていたが，ようやく12月5日の時点で少なくとも2種類の診断キットが入手可能になった。ひとつはELISA (Genelabs Diagnostic)で，もうひとつはイムノクロマト（Genelabs Diagnostic)，どちらもSARSコロナウイルス特異的な抗原（2種類のリコンビナントタンパク質ー種類などは現時点では公表されていない）に対するIgGを検出するキットである。急性

期血清はSARSが疑われた時点で即座に，回復期血清は発症20日以降に採取する。血液は血清に分離した後，それぞれ血清で1～2mL程度が必要である。できれば，1週間毎に血清を保存し，可能な限り多くの病日の検体を採取・保存する必要がある。

抗原抗体反応を利用して，検体中のウイルス抗原を同定することができる。イムノクロマトグラフィーと呼ばれる簡便な方法を応用することで，インフルエンザ抗原や結核抗原などの検出キットが開発され，医療現場でよく使われている。SARSの場合，これまでのところ抗原検出用の診断法の報告はない。今後は，遺伝子診断と共に必要な検査法になるであろう。

Ⅶ 今後の展望

今冬SARSが再び流行するかどうかはっきりしないが，ゲノム情報の蓄積にもとづいた種々の遺伝子診断法が開発され，感染研と地衛研のネットワークを中心に，現時点での標準的なSARS診断がいつでも可能な体制となっている[6]。しかし，いくつかの課題がある。第一に，SARSの早期診断が可能な，より高感度な遺伝子診断法の開発が急務である。また，遺伝子診断法に比べ，抗体検査法の開発が遅れている。この方面でも産官学が共同で抗体検査法を開発する必要がある。

文　献

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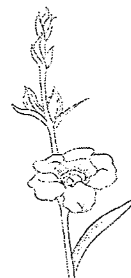
2) WHO：WHO guidelines/recommendations/descriptions Severe Acute Respiratory Syndrome (SARS)；http：//www.who.int/csr/sars/guidelines/en/
3) WHO：WHO研究施設ネットワークが集積したSARSコロナウイルスの安定性と抵抗性に関する最初のデータ；http：//idsc.nih.go.jp/others/urgent/update56-data.html
4) 国立感染症研究所ウイルス第三部：SARSコロナウイルス検出のためのウイルス分離用検体の採取・処理法およびウイルス分離；http：//idsc.nih.go.jp/others/urgent/update56-c.html
5) 国立感染症研究所ウイルス第三部：RT-PCR法によるSARSコロナウイルス遺伝子の検出；http：//idsc.nih.go.jp/others/urgent/update99-PCR.html
6) 国立感染症研究所感染症情報センター：今冬のSARSについて；http：//idsc.nih.go.jp/others/urgent/sars03w/home.html

＊ 基準となる検査法に関しては，文献5と6から検索可能である。



V. 参 考 資 料

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Production of cattle lacking prion protein

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Prion diseases are caused by propagation of misfolded forms of the normal cellular prion protein PrP^{C}, such as PrP^{BSE} in bovine spongiform encephalopathy (BSE) in cattle and PrP^{CJD} in Creutzfeldt-Jakob disease (CJD) in humans[1]. Disruption of PrP^{C} expression in mice, a species that does not naturally contract prion diseases, results in no apparent developmental abnormalities[2–5]. However, the impact of ablating PrP^{C} function in natural host species of prion diseases is unknown. Here we report the generation and characterization of PrP^{C}-deficient cattle produced by a sequential gene-targeting system[6]. At over 20 months of age, the cattle are clinically, physiologically, histopathologically, immunologically and reproductively normal. Brain tissue homogenates are resistant to prion propagation *in vitro* as assessed by protein misfolding cyclic amplification[7]. PrP^{C}-deficient cattle may be a useful model for prion research and could provide industrial bovine products free of prion proteins.

To generate PrP^{C}-deficient (*PRNP*$^{-/-}$) cattle, we transfected a male Holstein primary fetal fibroblast line 6594 with first and second knockout (KO) vectors (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors)[6] to sequentially disrupt the two alleles of *PRNP*. *PRNP*$^{-/-}$ fetal cell lines were established at 40–60 d of gestation and three of the *PRNP*$^{-/-}$ fetal cell lines (5211, 5232 and 4296) were recloned to produce calves (**Table 1** and **Fig. 1a**). To verify that the calves possess the *PRNP*$^{-/-}$ genotype, we collected ear biopsies and established fibroblast cell lines for genotyping. Genotyping was done by genomic PCR specific to each gene targeting event[6] (primer pairs: neoF7 × neoR7 and puroF14 × puroR14, **Fig. 1b**), followed by sequence analysis. Negative PCR analysis[6] was also carried out to confirm the absence of wild-type *PRNP* alleles (primer pairs: BPrPex3F × BPrPex3R, **Fig. 1b**). All calves born were *PRNP*$^{-/-}$.

To demonstrate functional inactivation of the *PRNP* gene in these calves, we extracted mRNA and protein from the *PRNP*$^{-/-}$ fibroblasts. Wild-type calves served as controls. For mRNA expression analysis, we performed RT-PCR[6] (primer pairs: PrPmF3 × PrPmR3, **Fig. 1c**) and confirmed the disruption of *PRNP*-specific mRNA expression in *PRNP*$^{-/-}$ calves. For protein expression analysis, we performed PrP-specific western blot analyses on fibroblasts (**Fig. 1d**), peripheral blood lymphocytes (**Fig. 1e**) and brain stem (**Fig. 1f**) from wild-type and *PRNP*$^{-/-}$ calves using the mouse anti-bovine PrP monoclonal antibody F89. We detected PrP-specific bands in the wild-type calves, whereas no reaction was observed in *PRNP*$^{-/-}$ calves and negative control mouse fibroblasts. These data clearly demonstrate that the *PRNP* gene is functionally inactivated in the *PRNP*$^{-/-}$ calves.

PRNP$^{-/-}$ cattle were monitored for growth and general health status from birth to 20 months of age. Mean birth weight was 46 kg and average daily gain was 0.91 kg/d to 10 months. Both values were in the normal range for Holstein bulls. Serum chemistry was evaluated at 6 months of age and compared with published reference ranges. All the values for *PRNP*$^{-/-}$ calves ($n = 12$) were well within the reference range (**Supplementary Table 1**) and obvious abnormalities were not observed. General physical examinations included the following parameters: body temperature, heart rate, heart sound, jugular vein distension, respiratory rate, respiratory sound, presence of cough, nasal discharge, eye abnormalities, appetite, general behavior (alert and active, sluggish, hyperactive), gait, posture, joints, hooves, feces (diarrhea, constipation) and genitalia and umbilical cord (dry, enlarged, inflamed, infected). All parameters were normal for all *PRNP*$^{-/-}$ cattle ($n = 12$).

At 10 months of age, eight pairs of *PRNP*$^{-/-}$ and age-, sex- and breed-matched wild-type control cattle were given an extensive clinical examination (consisting of 122 parameters). These examinations were done according to the diagnostic evaluation of ruminants suspected of transmissible spongiform encephalopathy (TSE) as described in the European TSE guideline "Surveillance and diagnostic of TSEs in ruminants"[8,9]. The clinical evaluation included a general examination of all organic systems and a detailed examination of the nervous system. Examination of the nervous system was focused on the following aspects: (i) evaluation of mental status, studied by observation of animal behavior and reactions to stimulation (approaching, menace, sounds and light); (ii) evaluation of sensory function in limbs and trunk, including study of superficial sensitivity, medular reflexes and conscious proprioception; (iii) evaluation of motor function in limbs and trunk by studying muscular tone, motor irritability (presence of muscle fasciculation and tremor) and gait abnormalities

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Table 1 Production of cloned calves from *PRNP*$^{-/-}$ fibroblast cell lines

Cell line ID	Embryos implanted	Recipients	Pregnant at (%)[a]				Live animals at 6 months (%)[a]
			40 d	90 d	150 d	270 d	
5211	45	30	17 (38)	7 (16)	7 (16)	6 (13)	5 (11)
5232	21	21	7 (33)	4 (19)	4 (19)	3 (14)	2 (10)
4296	19	19	9 (47)	7 (37)	6 (32)	5 (26)	5 (26)
Total	85	70	33 (47)	18 (26)	17 (24)	14 (20)	12 (14)

[a]Percentages were calculated by dividing the number of fetuses or calves by that of embryos implanted.

and, finally, (iv) evaluation of cranial nerves by observation of disorders in the corresponding innervated regions.

All animals (*PRNP*$^{-/-}$ and control cattle) appeared a healthy in the general clinical examination. The nervous system evaluation revealed little change other than a mild increased reaction to external stimulation (menace and sounds) in 3/8 *PRNP*$^{-/-}$ cattle compared to 1/8 control cattle.

Blood samples were taken for hematological analysis from five pairs of *PRNP*$^{-/-}$ and control cattle matched for age-, sex- and breed, at 10 and 12 months of age. The means for various hematological parameters from the two samples were compared between *PRNP*$^{-/-}$, control cattle and published reference ranges (**Supplementary Table 2**). *PRNP*$^{-/-}$ cattle had slightly lower values for mean corpuscular volume and mean corpuscular hemoglobin compared to wild-type cattle; both groups were low compared to reference values. However, other measures of erythrocyte characteristics were normal for both groups. *PRNP*$^{-/-}$ cattle had higher values for white blood cell and neutrophil counts compared to controls, but values for both groups were well within the reference range. Overall, hematological analysis did not reveal obvious unusual characteristics in *PRNP*$^{-/-}$ cattle at 10 or 12 months of age, but further study will be necessary to determine whether the slight differences observed in the knockout cattle might be attributed to disruption of *PRNP* gene function or the presence of the knockout cassettes.

The normal prion protein, PrP^{C}, is most abundantly expressed in the central nervous system (CNS) and lymphoid cells, and the propagation and accumulation of PrP^{BSE} in the CNS leads to neurodegeneration and prion disease[10,11]. To evaluate the impact of PrP^{C} deletion on calf development, we carried out extensive gross and histopathological analyses on two *PRNP*$^{-/-}$ and two age-, sex- and breed-matched wild-type cattle at 14 months of age. Representative samples of tissues were evaluated by gross and microscopic examinations. The two groups of cattle were of approximately similar body weights, their carcasses were in good nutritional condition, and no significant lesions were observed on gross examination of organs. At least 14 sections of various areas of the brain (including obex, pons, colliculi, cerebellum, hippocampus, thalamus and cerebral cortex) of each animal were examined by light microscopy. Two sections of spinal cord at cervical, thoracic and lumbar regions were also evaluated by light microscopy. No obvious abnormalities or significant lesions were observed in any tissues in either of the two groups. In particular, no plaques of spongiform tissue or neurodegeneration were detected in the obex and cerebellum of *PRNP*$^{-/-}$ cattle (**Fig. 2**). The cerebellar Purkinje cells of the *PRNP*$^{-/-}$ animals at 14 months of age showed no evidence of cell loss (**Fig. 2d**).

Cells of the immune system play an important role in the pathogenesis of prion diseases, and PrP^{C} expression is readily detected in immune cells[12,13]. Therefore, we examined the effects of PrP^{C} deficiency on the immune system of *PRNP*$^{-/-}$ cattle at 12–13 months of age. We evaluated B-cell and T-cell populations in peripheral blood lymphocytes (PBLs) of *PRNP*$^{-/-}$ cattle by flow cytometry. No significant differences were observed in any of these cell subsets between *PRNP*$^{-/-}$ and age-, sex- and breed-matched wild-type cattle (**Fig. 3a–d**; statistical comparison is provided in **Supplementary Table 3**).

In *Prnp*$^{-/-}$ mice, T-cell proliferation and cytokine production induced by T-cell mitogens are significantly affected, suggesting a role of PrP^{C} in T-cell function[14,15]. Therefore, PBLs were isolated from *PRNP*$^{-/-}$ cattle and stimulated with anti-CD3 antibody, concanavalin A and phytohemagglutinin (PHA). In contrast to *Prnp*$^{-/-}$ mice, no

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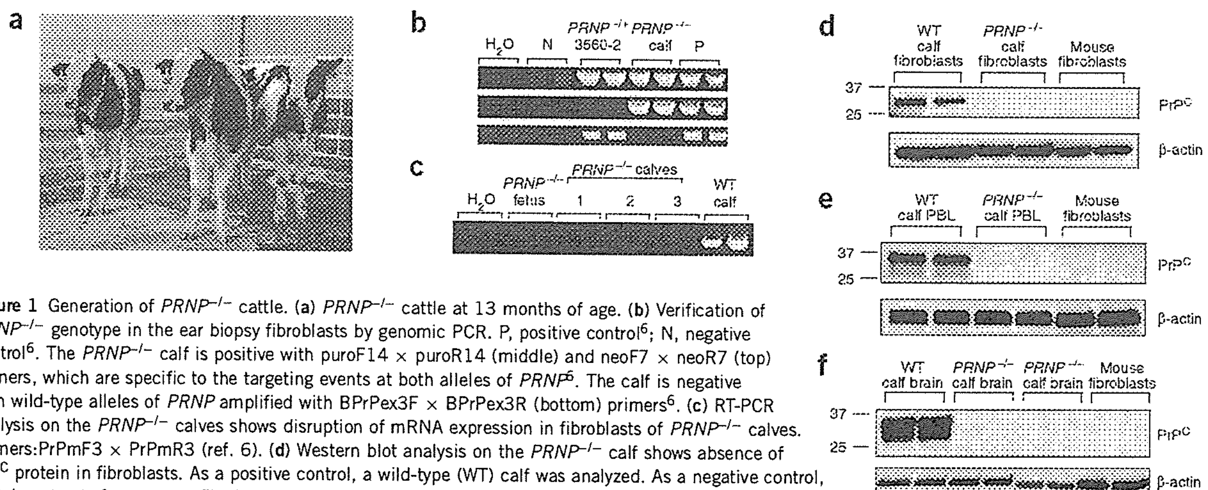

Figure 1 Generation of *PRNP*$^{-/-}$ cattle. (**a**) *PRNP*$^{-/-}$ cattle at 13 months of age. (**b**) Verification of *PRNP*$^{-/-}$ genotype in the ear biopsy fibroblasts by genomic PCR. P, positive control[6]; N, negative control[6]. The *PRNP*$^{-/-}$ calf is positive with puroF14 × puroR14 (middle) and neoF7 × neoR7 (top) primers, which are specific to the targeting events at both alleles of *PRNP*[6]. The calf is negative with wild-type alleles of *PRNP* amplified with BPrPex3F × BPrPex3R (bottom) primers[6]. (**c**) RT-PCR analysis on the *PRNP*$^{-/-}$ calves shows disruption of mRNA expression in fibroblasts of *PRNP*$^{-/-}$ calves. Primers:PrPmF3 × PrPmR3 (ref. 6). (**d**) Western blot analysis on the *PRNP*$^{-/-}$ calf shows absence of PrP^{C} protein in fibroblasts. As a positive control, a wild-type (WT) calf was analyzed. As a negative control, protein extracts from mouse fibroblasts were used because the monoclonal antibody used is claimed to be specific to bovine PrP^{C} protein. Protein extracts from wild-type calf show the presence of 33–35 kDa of bovine PrP^{C} protein in size, but no positive band from the *PRNP*$^{-/-}$ calf. Its replica blot was probed with anti-β actin antibody and served as an internal positive control. (**e**) Absence of PrP^{C} protein in peripheral blood lymphocytes (PBLs) of *PRNP*$^{-/-}$ calf by western blot analysis. (**f**) Absence of PrP^{C} protein in brain stem of *PRNP*$^{-/-}$ calves by western blot analysis.

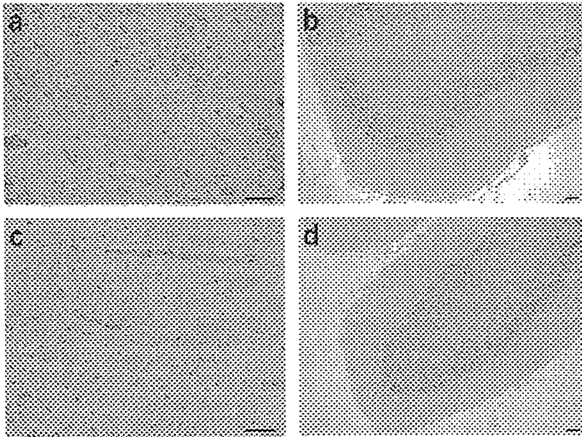

Figure 2 Histopathological analysis of obex and cerebellum of 14-month-old cattle. (**a**,**b**) *PRNP*$^{+/+}$ dorsal motor nucleus of vagus (**a**) and molecular layer, granular layer and white matter (**b**). (**c**,**d**) *PRNP*$^{-/-}$ dorsal motor nucleus of vagus (**c**) and molecular layer, granular layer and white matter (**d**). There are neither plaques of spongiform tissues nor apparent neurodegeneration in the tissues. H & E stain. Scale bars, 100 μm.

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significant difference in T-cell proliferation after T-cell mitogen stimulation was observed for *PRNP*$^{-/-}$ cattle as compared to similarly treated cells from wild-type cattle (statistical analysis using Student's *t*-test: anti-CD3, $P = 0.9$; Con A, $P = 0.4$; PHA, $P = 0.7$) (**Fig. 3f**). In addition, no obvious difference between the two groups of cattle for intracellular and secreted interferon-γ (IFNγ) production was observed after T-cell mitogen stimulation (**Fig. 3g,h**). Finally, to address immune competence of *PRNP*$^{-/-}$ cattle *in vivo*, we immunized them with ovalbumin, a T cell–dependent antigen. The ovalbumin-specific humoral immune response in *PRNP*$^{-/-}$ cattle was similar to that of the controls (**Fig. 3i**). Collectively, these data indicate that ablation of PrP^C expression does not appear to have deleterious effects on the immune systems of cattle.

The *PRNP*$^{-/-}$ bulls reached sexual maturity at a normal age and semen was collected from two knockout animals at 16 months of age. Sperm appeared morphologically normal (**Supplementary Fig. 1a**) and were capable of generating normal-appearing blastocysts (**Supplementary Fig. 1b**) by *in vitro* fertilization (IVF) with oocytes derived from wild-type cows at an efficiency similar to that of control IVF (**Supplementary Table 4**). Twelve blastocysts were implanted and eight cows were pregnant at 40 d of gestation. This result indicates that *PRNP*$^{-/-}$ sperm appears to be reproductively normal. Future studies will determine whether the blastocysts can produce normal offspring.

To determine whether the absence of endogenous bovine PrP^C indeed prevents PrP^{BSE} propagation *in vitro*, we collected two brain regions (cortex and hypothalamus) from 10-month-old *PRNP*$^{-/-}$ cattle for a protein misfolding cyclic amplification (PMCA) assay[7,16–18]. As control substrate for the PMCA assay, CNS tissues from the identical anatomic sites were obtained from an age-, sex- and breed-matched wild-type calf. PMCA was carried out with brain homogenates derived from either *PRNP*$^{-/-}$ or wild-type cattle as 'PMCA substrates'; a brain homogenate from a BSE-infected cow was used as the PrP^{BSE}-containing inoculum. No propagation of proteinase K (PK)-resistant PrP^{BSE} was detected by western blot analysis when brain homogenates from the *PRNP*$^{-/-}$ cattle were used as substrates (**Fig. 4a,b**). In contrast, PrP^{BSE} was readily amplified and detected in western blot analysis when brain homogenates from the wild-type cattle were used as substrates (**Fig. 4a,b**). These results indicate that the presence of endogenous bovine PrP^C is essential for PrP^{BSE} propagation *in vitro* and that any other host-derived cellular cofactors included in the brain homogenates, such as RNA[19] and sulfated glycosaminoglycan[20], cannot support the *in vitro* PrP^{BSE} propagation in the absence of endogenous bovine PrP^C. We also performed a similar PMCA assay using a brain homogenate from cattle infected with transmissible mink encephalopathy (TME), another prion strain infectious to cattle, as inoculum and detected no propagation of the PrP^{TME} (**Fig. 4c**). This indicates that *PRNP*$^{-/-}$ cattle could be resistant to propagation of various prion strains.

In summary, we have demonstrated the usefulness of the sequential gene targeting system to efficiently produce *PRNP*$^{-/-}$ cattle. Excluding three animals sacrificed for the histopathological analysis and PMCA assay, all the nine *PRNP*$^{-/-}$ cattle have remained healthy for at least 20 months after birth without showing obvious clinical abnormalities. This indicates that 'loss of function' of bovine PrP^C itself does not cause BSE and that ablation of the normal cellular prion protein PrP^C function does not adversely affect normal bovine development. It has been reported that evolution has exerted very intense purifying selection on exon 3 of bovine *PRNP*; the *PRNP* gene should have some indispensable function in bovine development because such strong purifying selection is usually seen only for proteins essential to eukaryotic life[21]. Therefore, our findings appear to be of particular interest in supporting a general hypothesis that PrP^C function is dispensable for normal animal development.

Moreover, brain homogenates from *PRNP*$^{-/-}$ cattle were resistant to the *in vitro* propagation of at least two different prion strains, PrP^{BSE} and PrP^{TME}, by the PMCA method. PMCA has been shown to closely mimic *in vitro* the prion propagation process that occurs *in vivo*, leading to the formation of high quantities of misfolded prion protein that are infectious to wild-type animals[16]. PMCA is described to be at least as sensitive to prion propagation as *in vivo* infection[22] and reproduces the species barrier and prion strain phenomenon typical of the prion infectious agent (J.C. and C.S., unpublished data).

In *Prnp*$^{-/-}$ mice, phenotypes vary depending on how the *Prnp* gene locus is disrupted. For example, ataxia and loss of Purkinje cells in aged mice have been observed in some *Prnp*$^{-/-}$ mouse strains where, in addition to the *Prnp* open reading frame (ORF), 5′ flanking genomic sequences were also deleted, which results in exon skipping between *Prnp* and *Prnd* (located 16 kb downstream of the murine gene *Prnp* and encoding Dpl protein)[23–27]. The cerebellar symptoms in these *Prnp*$^{-/-}$ mouse strains were suggested to be caused by ectopic expression of Dpl protein in brain[28]. In the *PRNP*$^{-/-}$ cattle described here, only the ORF of the bovine *PRNP* gene was disrupted by insertion of the *neo* and *puro* cassettes without any deletion of *PRNP* genomic sequences, so that any splicing donor/acceptor sites remain intact[6]. It remains to be determined whether or not the bovine *PRND* locus might be affected by the disruption of *PRNP*, but if it occurs, its effect would appear to be minimal because obvious abnormalities, such as ataxia and Purkinje cell loss, were not found in *PRNP*$^{-/-}$ cattle up to 20 months of age.

Consistent with our observations in the *PRNP*$^{-/-}$ cattle, *Prnp*$^{-/-}$ mice with exclusive disruption on the *Prnp* ORF remained healthy[2,3] and showed only slightly abnormal phenotypes, such as altered synaptic function[29,30] and sleep-wake circadian rhythms[31,32]. However, the phenotype in the synaptic function appears to be normal in other murine genetic backgrounds[33]. We have monitored sleep-wake activity in the knockout cattle, along with age-, sex- and breed-matched wild-type controls, at frequent intervals throughout the day and night for

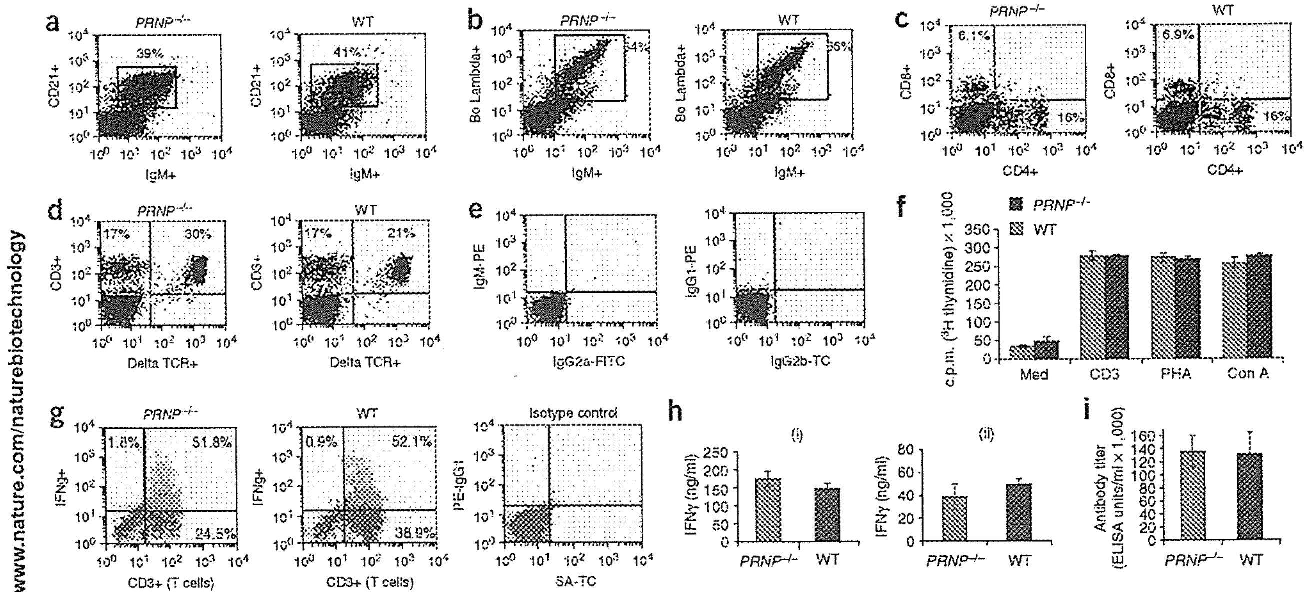

Figure 3 Comparative analysis of immune system of $PRNP^{-/-}$ and wild-type (WT) control cattle at 12–13 months old. (a) Flow cytometry in peripheral blood lymphocyte (PBL), stained with anti-IgM and anti-CD21 antibodies. (b) PBLs stained with anti-IgM and anti-lambda light-chain antibodies. (c) PBLs stained with anti-CD4 and anti-CD8 antibodies. (d) PBLs stained with anti-CD3 and anti-γδ T cell–receptor antibodies. (e) Secondary antibody isotype control staining. (f) *In vitro* mitogenic response of T cells in $PRNP^{-/-}$ and WT cattle. PBLs from four $PRNP^{-/-}$ and four WT cattle were cultured with medium only (Med) or stimulated with immobilized anti-CD3 monoclonal antibody (CD3), Con A (concanavalin A) or PHA (phytohemagglutinin) mitogens for 48 h and proliferation was measured by ^{3}H thymidine incorporation. Mean of T-cell response of $PRNP^{-/-}$ group and WT group cattle and their s.e.m. are shown. No significant differences were found. (g) Intracellular cytokine analysis of IFNγ expression in $PRNP^{-/-}$ and WT control cattle by dual-color flow cytometry. PBLs were stimulated by immobilized anti-CD3 monoclonal antibody for 72 h and intracellular IFNγ production was analyzed by surface CD3 and intracellular IFNγ (positive, green; negative, red) dual-color immunofluorescent staining. Percentage of IFNγ$^+$ T cells are shown in the upper right quadrant. (h) *In vitro* IFNγ production by PBLs in $PRNP^{-/-}$ and WT cattle. PBLs isolated from four $PRNP^{-/-}$ and four WT cattle were stimulated by (i) immobilized anti-CD3 monoclonal antibody or (ii) Con A for 72 h and secreted IFNγ in the culture supernatant was analyzed by calibrated bovine IFNγ ELISA. Mean of the IFNγ production (ng/ml) in $PRNP^{-/-}$ group and WT control cattle and their s.e.m. are shown. Statistical analysis using Student's *t*-test showed no significant difference between $PRNP^{-/-}$ and WT cattle ($P = 0.5$). (i) Humoral immune response to ovalbumin protein antigen in $PRNP^{-/-}$ and WT cattle. Four $PRNP^{-/-}$ and four WT cattle were immunized with ovalbumin twice at day 0 (V1) and day 21 (V2) and ovalbumin-specific IgG antibody titers at 7 d after V2 were determined. Mean antibody titers of $PRNP^{-/-}$ group and WT group cattle and their s.e.m. are shown. Statistical analysis using Student's *t*-test showed no significant difference between $PRNP^{-/-}$ and WT cattle ($P = 0.9$).

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one week, but did not observe any obvious alterations. Some other $Prnp^{-/-}$ mouse models show subtle abnormalities, such as learning differences[34], and deletion of parts of the murine *Prnp* ORF have more severe effects[35], suggesting that complete ablation of PrP^C function as done in this study may have less detrimental phenotypes than the partial manipulation or rearrangement of the *Prnp* ORF.

$PRNP^{-/-}$ cattle are likely a more relevant model for elucidating PrP^C function and the basic mechanisms of prion pathogenesis than mice, as cattle are a natural host of prion diseases. In particular, $PRNP^{-/-}$ cattle allow *in vivo* tests of resistance to prion propagation. We have undertaken such tests, which will require at least 3 years to complete.

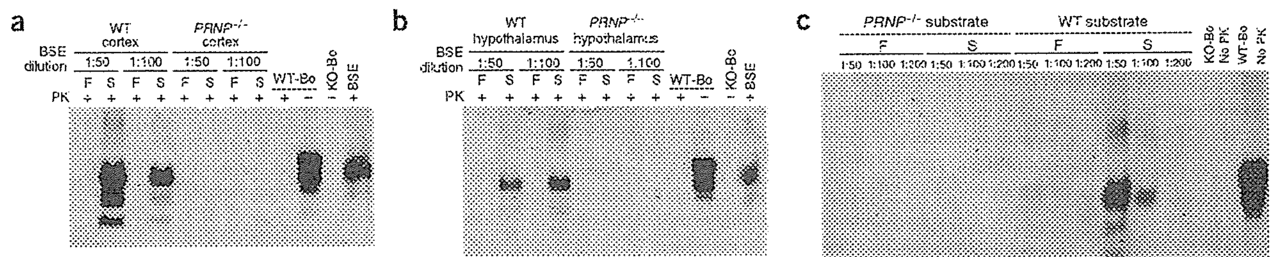

Figure 4 *In vitro* propagation of PrP^{BSE} and PrP^{TME} in $PRNP^{-/-}$ and $PRNP^{+/+}$ wild-type (WT) cattle brain homogenates. (a,b) *In vitro* propagation of PrP^{BSE} in 10% homogenates from cortex (a) or hypothalamus (b). (c) *in vitro* propagation of PrP^{TME} in 10% homogenates from cortex (c). The pathological form of the prion protein, PrP^{BSE} or PrP^{TME}, in the inoculum, was derived from BSE- or TME-affected cattle, respectively. We used 1:50, 1:100 and 1:200 dilutions of the infectious material. Samples were either frozen immediately after mixture (F) or subjected to 48 PMCA amplification cycles (S). The appearance of PrP^{BSE} or PrP^{TME} was assessed by western blot analysis after proteinase K (PK) digestion. Samples from $PRNP^{+/+}$ wild-type (WT-Bo), $PRNP^{-/-}$ (KO-Bo) cattle (substrates) and the BSE-positive control brain homogenate (inoculum) are shown for comparison with and without PK treatment.

PRNP$^{-/-}$ cattle could be a preferred source of a wide variety of bovine-derived products that have been extensively used in biotechnology, such as milk, gelatin, collagen, serum and plasma. In addition to the *PRNP*$^{-/-}$ cattle described here, we have also generated healthy *PRNP*$^{-/-}$*IGHM*$^{-/-}$ (ref. 6) double-knockout cattle (**Supplementary Fig. 2**). This indicates that other genetic modifications can be added to the *PRNP*$^{-/-}$ background by means of consecutive rounds of gene modification and recloning, alleviating time-consuming breeding of livestock. Additional genetic modifications to *PRNP*$^{-/-}$ background could be useful for production of prion protein–free therapeutic recombinant human proteins, tissue and organs in transgenic livestock for biomedical applications.

Although a ban on feeding cattle ruminant-derived meat-bone meal has greatly reduced BSE infections in cattle, the possibility cannot be completely excluded that some PrP^{BSE} strains might have originated from 'spontaneous' misfolding of the endogenous PrP^{C} protein. This view is supported by recent reports suggesting the presence of atypical PrP^{BSE} strains[36–38]. Two cattle recently identified in the United States (Texas and Alabama) appear to show an atypical PrP^{BSE} pattern, and one animal with atypical PrP^{BSE} characteristics was born after the feed ban[37]. Thus, the ban may not completely alleviate concerns about BSE. The *PRNP*$^{-/-}$ cattle produced in this study would prevent BSE due to spontaneous misfolding because of a complete lack of endogenous PrP^{C}.

METHODS

Embryonic cloning. Cloned fetuses and calves were produced using the chromatin transfer procedure as described[6]. *In vitro* embryo development rate is provided in **Supplementary Table 5**. All animal work described in this section was done following a protocol approved by the Transova Genetics Institutional Animal Care and Use Committee.

Generation of *PRNP*$^{-/-}$ fetal cell lines. Sequential gene targeting was carried out as described previously[6]. Two types of knockout vectors were used (pBPrP(H)KOneo and pBPrP(H)KOpuro vectors) to sequentially disrupt both alleles of *PRNP*. To provide more specific information on the bovine *PRNP* genomic DNA, we used ~8.3 kb of *Bam*HI-*Bam*HI region (base position 65605-73896 of GenBank accession no. AJ298878) for the 3′ homologous arm and ~1.2 kb of *Bgl*II-*Bam*HI region (base position 64494-65604 of AJ298878) for the 5′ homologous arm. Male Holstein primary fetal fibroblast line 6594 was electroporated at 550 V and 50 μF with the first knockout vector (pBPrP(H)KOneo). We screened 94 colonies resistant to G418 (500 μg/ml) by PCR to identify homologous recombinants (primer pair; neoF7 × neoR7) and then homologous recombinants were identified (40/94: 43%). Based on their morphology, we selected seven colonies and cloned embryos to generate fetuses. At 40–60 d of gestation, five fetuses were collected and three of them (2180, 3560-1 and 3560-2) were confirmed to be *PRNP*$^{-/+}$ (primer pair; neoF7 × neoR7). The heterozygous *PRNP*$^{-/+}$ cell line, 3560-2, was electroporated with the second knockout vector (pBPrP(H)KOpuro), and 182 colonies resistant to puromycin (1 μg/ml) were screened by PCR (primer pair; puroF14 × puroR14) to identify homozygously targeted colonies. Six colonies were identified to be *PRNP*$^{-/-}$, four of which were used for embryonic cloning to generate recloned fetuses. At 40–75 d of gestation, ten fetuses were collected and then fibroblast cell lines were established. All of them were confirmed to be homozygous *PRNP*$^{-/-}$ by the targeting event-specific (puroF14 × puroR14 and neoF7 × neoR7) and negative (BPrPex3-F x BPrPex3-R) PCR analyses. We also performed Southern hybridization analysis on *Sph*I and *Bam*HI-double digested genomic DNA extracted from *PRNP*$^{-/-}$ fibroblast cell lines using the coding region of the *neo* or *puro* gene as a probe, which showed the expected band size and a single-site integration of the knockout cassettes.

RT-PCR. RNA was extracted from *PRNP*$^{+/+}$ and *PRNP*$^{-/-}$ calf fibroblasts by using RNeasy mini kit (Qiagen), and first strand cDNA synthesis was done by using superscript first-strand synthesis system for RT-PCR (Invitrogen). PCR was done as previously described[6].

Western blotting. Protein was extracted from *PRNP*$^{+/+}$ and *PRNP*$^{-/-}$ calf fibroblasts, peripheral blood lymphocytes and brain stem. The protein content was quantified with Bio-Rad protein assay reagent. Western blot analysis was carried out by running ~75 μg of protein sample on a 12% SDS-PAGE gel under nonreducing conditions. The proteins were transferred to a nitrocellulose membrane and the membrane was stained by the mouse anti-bovine prion protein monoclonal antibody (F 89/160.1.5 from Alexis Biochemicals) as a primary antibody and, second, stained with peroxidase-labeled affinity-purified antibody to mouse IgG (H+L). The stained membrane was developed by ECL plus western blot analysis detection system (Amersham Bioscience) and exposed to Biomax light film by film developer. Detection limit for PrP^{C} protein in this western blot analysis was estimated to be ~1.2 μg of brain homogenate from the wild type, which was ~60-fold less than the protein amount (75 μg total brain protein) used in **Figure 1**.

Ovalbumin immunization. Four *PRNP*$^{-/-}$ and four control wild-type calves 12–13 months old were immunized with ovalbumin antigen (Sigma) at 1 mg/dose formulated with Montanide ISA 25 adjuvant (Seppic) as water-in-oil emulsion. The calves were immunized twice at 3-week intervals (primary immunization followed by first booster after 3 weeks). Vaccine was administered by intramuscular injection (2-ml dose containing 1 mg/ml ovalbumin plus 1 ml of ISA-25 adjuvant) in the neck region. Serum samples were collected before each immunization (V1 and V2) and 7 d and 14 d after each immunization for antibody titer analysis. Blood was drawn into serum separator tubes (tiger-top), allowed to clot and serum was separated by centrifugation. Serum was then divided into 0.5- to 1-ml aliquots and stored frozen until assays were performed. Anti-ovalbumin antibody titers were determined by ovalbumin-specific IgG enzyme-linked immunosorbent assay (ELISA).

Flow cytometry. Peripheral blood was collected from four *PRNP*$^{-/-}$ and four control wild-type calves 12–13 months old by jugular venipuncture into heparinized tubes. Whole white blood cells (leukocytes) were isolated from heparinized blood using red blood cell lysis (RBC-lysis buffer from Sigma) followed by two washes with PBS. Sheep anti-bovine IgM-FITC (Bethyl Laboratories) was used to label bovine surface IgM (sIgM) on the B cells. Mouse anti-bovine CD21 (Clone MCA1424 from Serotec) antibody or anti-bovine lambda light chain (Clone: BIG501E, VMRD) followed by anti-mouse IgG1-PE secondary antibody (Caltag Laboratories) was used to label surface CD21 marker on bovine B cells. For T-cell analysis, anti-CD3 (Clone MM1A, VMRD), anti-CD4 (Clone IL-A11, VMRD), anti-CD8 (Clone BAQ 111A, VMRD) and anti-γδ TCR (Clone GB21A, VMRD) monoclonal antibodies were used, followed by fluorochrome labeled isotype-specific secondary antibodies (IgG1-PE; IgG2a-FITC; IgM-PE; IgG2b-TC, purchased from Caltag). Staining was done by a standard protocol and ~10,000 gated lymphocytes were analyzed by FACScan flow cytometer (BD Biosciences).

***In vitro* T-cell responses to mitogen stimulation.** Heparinized blood was collected from four *PRNP*$^{-/-}$ and four age-, breed- and sex-matched wild-type control calves 12–13 months old, and PBLs were isolated using Ficoll gradient centrifugation. After three washes with sterile HBSS, cells were resuspended in complete RPMI medium (Sigma) with 10% FBS (Hyclone) and cultured with medium only (control), 5 μg/ml Con A (Sigma), 2.5 μg/ml PHA (Sigma) or 5 μg/ml purified anti-CD3 mAb (GB21-A from VMRD) immobilized on culture wells. Separate cultures were set up in triplicate microtiter wells or 48-well plate for proliferation and cytokine assays. For proliferation assays, cultures were pulsed at 48 h and 72 h with 0.5 μCi of ^{3}H thymidine (Amersham BioSciences) for 4 h and incorporation of ^{3}H thymidine (proliferation) was measured by liquid scintillation counter as c.p.m. units. For IFN-γ ELISA, cultures were left for 72 h and culture supernatant was analyzed for secreted IFN-γ protein using calibrated Bovine IFN-γ ELISA Kit (MabTech). Intracellular IFN-γ production in T cells was measured by dual-color intracellular immunofluorescent staining. Brefeldin and Monensin (Sigma) were added to 48-well cultures at 66 h to stop secretion of cytokines and cells were cultured for a further 6–8 h. Cells were harvested and stained for surface CD3 marker using anti-CD3 monoclonal antibody (GB21-A from VMRD). Cells were then fixed, permeabilized and stained by anti- IFN-γ-Biotin antibody and

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Streptavidin-Tricolor for intracellular IFN-γ protein (MabTech). Approximately 10,000 gated lymphocytes were analyzed by FACSaria cell sorter (Becton Dickinson) and WinMDI software (Scripps Research Institute).

Gross and microscopic examinations. Four calves (2 wild-type and 2 *PRNP*$^{-/-}$ calves) were killed with pentobarbital and were subjected to complete necropsy examinations. Representative samples of skin, nasal turbinate, lung, liver, kidney, spleen, salivary gland, thyroid gland, tonsils (pharyngeal, palatine), thymus, reticulum, rumen, omasum, abomasum, intestines (ileum, colon), adrenal gland, pancreas, urinary bladder, lymph nodes (retropharyngeal, prescapular, mesenteric, popliteal), aorta, striated muscles (heart, tongue, masseter, diaphragm, triceps, psoas major, biceps femoris), testicle (from two animals), nictitating membrane, sciatic nerve, both trigeminal nerves and ganglia, pituitary gland, spinal cord (cervical, thoracic, lumbar), one eye with its optic nerve and the whole brain were evaluated by gross and microscopic examinations. The calves were of similar body weights and the mean ratio of brain weight (g)/body weight (kg) was 1.10 and 1.25 for *PRNP*$^{-/-}$ and the control cattle, respectively. The gyri of the knockout calves were slightly narrower when compared to the controls. The samples were immersion-fixed in 10% neutral buffered formalin. One eye with its optic nerve was immersion-fixed in 2% Bouin's fluid. The fixed brain was cut into 2- to 4-mm wide coronal sections for examination. The fixed tissues were processed for routine histopathology, embedded in paraffin wax, sectioned at 5 μm and stained with hematoxylin and eosin for examination by light microscopy. The examination was done by a board-certified, experienced bovine pathologist (A.N.H.) in side-by side comparisons of the tissues using multiple levels of magnification.

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***In vitro* fertilization.** Bovine cumulus oocyte complexes (COCs) collected from slaughter house ovaries and matured for approximately 24 h were fertilized in fertilization medium. Fresh semen collected from *PRNP*$^{-/-}$ bulls were prepared using Percoll gradient separation method and COCs were cultured with sperm (2 × 10^6 motile sperm/ml) for 18 h at 39 °C in an atmosphere of 5% CO_2 in air, after which they were stripped by vortexing in TL-HEPES for 2 min to remove the cumulus cells. Fertilized oocytes were cultured similarly to cloned embryos.

PMCA procedure. Ten percent brain homogenates (wt/vol) were prepared from the cortex or hypothalamus of either BSE or TME-affected animals, wild-type or *PRNP*$^{-/-}$ cattle. The homogenates were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, 4 mM EDTA and the complete protease inhibitor cocktail from Boehringer Mannheim). The samples were clarified by a brief, low-speed centrifugation (1,500 rpm for 30 s) using an Eppendorf centrifuge, model 5414. Dilutions of this brain homogenate were done in conversion buffer and they are expressed in relation to the brain, for example a 100-fold dilution is equivalent to a 1% brain homogenate. Aliquots of wild-type, *PRNP*$^{-/-}$ and BSE or TME brain homogenate prepared in conversion buffer were mixed and either immediately frozen or subjected to 48 cycles of PMCA. For PMCA, tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000) and programmed to perform cycles of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at 60% potency. The detailed protocol, including troubleshooting, has been recently published elsewhere[7,16–18,22]. Samples were incubated with 50 μg/ml of proteinase K (PK) for 60 min at 45 °C. The digestion was stopped by adding electrophoresis sample buffer. Proteins were fractionated by SDS-PAGE under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 antibody (Prionics) diluted 1:5,000 in PBS. The immunoreactive bands were visualized by enhanced chemiluminescence assay (Amersham).

Statistical analysis. Statistical analysis was performed by Student's *t*-test using both confidence interval estimate analysis and *t* score probability hypothesis testing method for two independent sample groups. Both methods of analysis showed that there were no significant differences between *PRNP*$^{-/-}$ and WT control cattle groups at α level of 0.05 and $P > 0.2$ or 0.1. However, there is a considerable probability of statistical error due to the limited numbers of subjects in this study.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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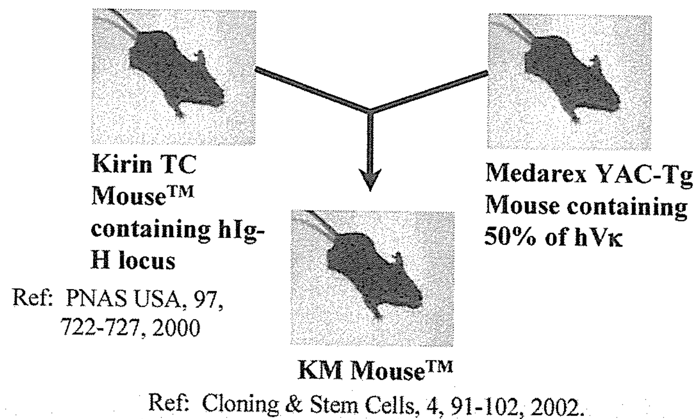
ヒト型抗体産生マウス及びウシ開発資料

1

Human Antibody Project

Kirin has established the platform for human monoclonal antibody business since Kirin formed alliance with Medarex to develop the best human antibody mouse in the world on January of 2000.



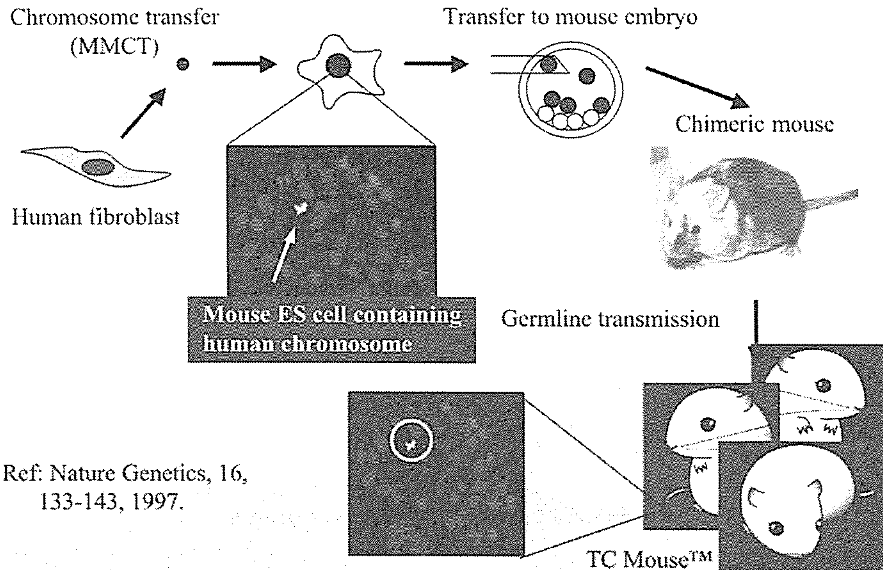
Ref: PNAS USA, 97, 722-727, 2000

Ref: Cloning & Stem Cells, 4, 91-102, 2002.

- Cross-licensing the IPs owned or controlled by Kirin and Medarex.

2

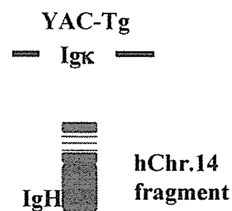
Generation of TC Mouse™



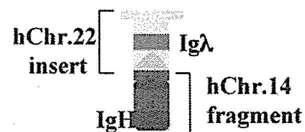
Ref: Nature Genetics, 16, 133-143, 1997.

Human Antibody Mouse

KM Mouse™
KM(FcγRIIb-KO)
KM(C3H,C57B6)
KM(H-2d)



HAC Mouse™
HAC-KM

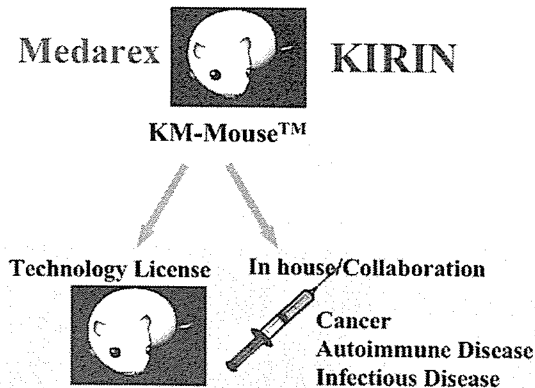


Ref: Nature Biotechnol., 20, 889-894, 2002 ; Gene Therapy, 9, 708-712, 2002.

4

Kirin Human Antibody Project

1. Kirin is developing human monoclonal antibody products in house and in collaboration mainly on cancer, immune-related and infectious disease area.
2. Kirin is licensing the human antibody mouse technology worldwide together with Medarex.

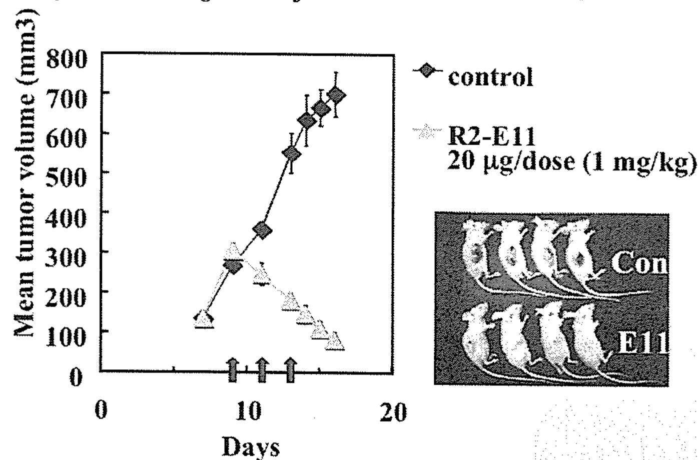


5

Development of Antibody Products

1. TRAIL-R2 human monoclonal antibody

Effect of R2-E11 on growth of 300mm³ Colo205 tumors

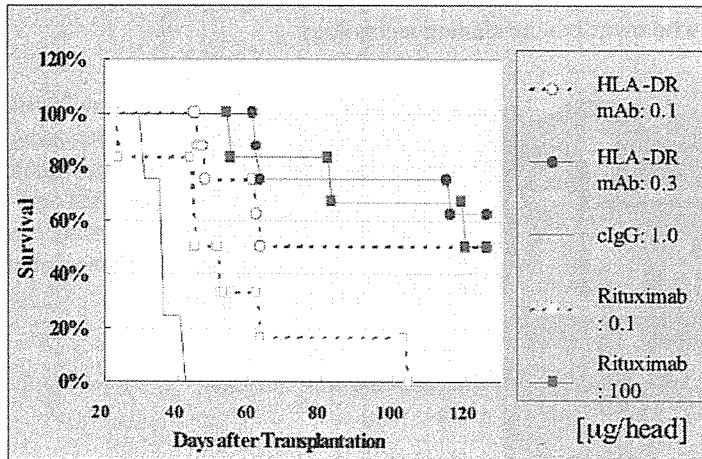


Ref: Cell Death and Differentiation, 11, 203-207, 2004.

6

Development of Antibody Products

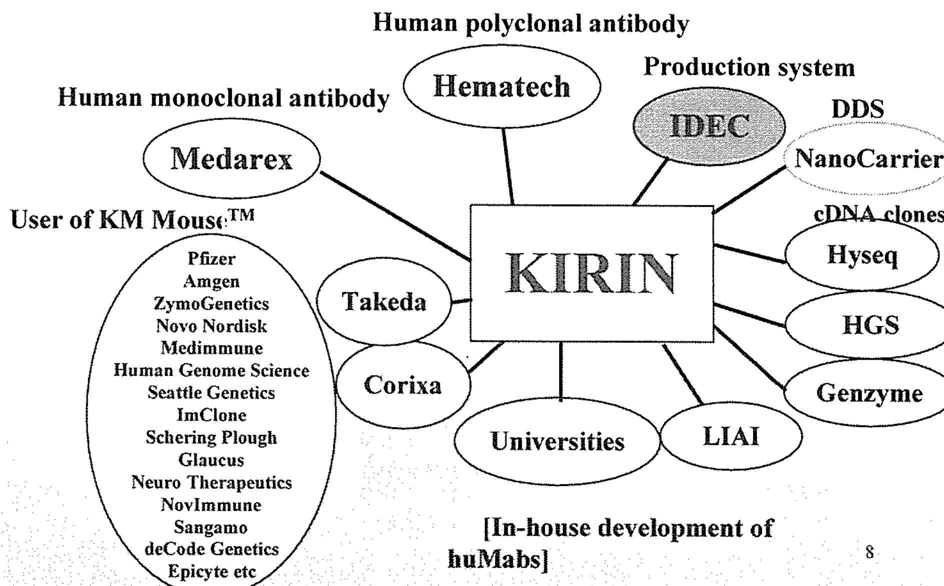
2. HLA-DR human monoclonal antibody



・Raji-xenografted scid mice were inoculated with HLA-DR mAbs 5 days after transplantation.

7

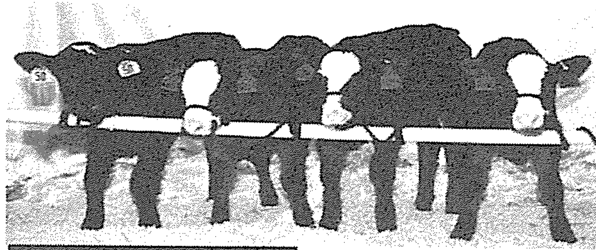
Licensing & Alliance



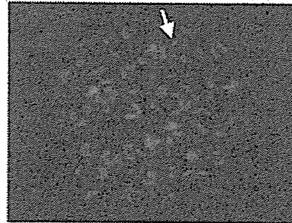
8

Kirin Human Antibody Project

Kirin has been developing human antibody producing cows in collaboration with Hematech who owns bovine cloning technology.



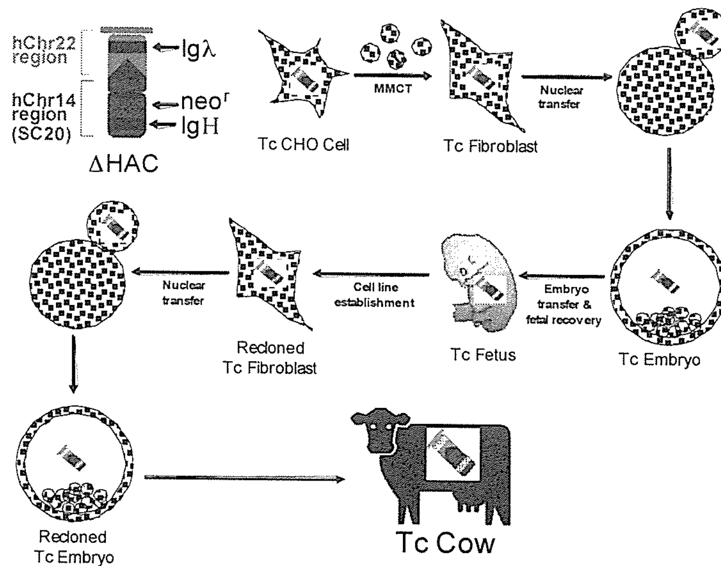
Cloned TransChromo Cow retaining a human chromosome fragment and expressing human immunoglobulin



A human chromosome in the Cloned TransChromo Cow

9

Generation of TC-cows



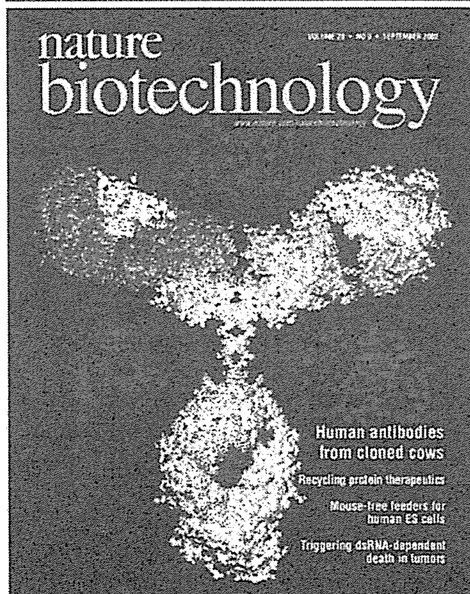
10

Kirin Human Antibody Project

We have generated more than 70 TC cows who express human immunoglobulin.
The TC cows express various levels of the human Igs in their blood.
Those TC cows produce antigen specific human Ig response upon immunization with the antigen.
Endogenous bovine Ig- and PrP-gene KOs are now in progress to improve the human immunoglobulin expression for commercialization of the antibody products.

11

Publication in Nature Biotechnology



100 Top Science Stories of 2002 Published in DISCOVER Vol. 24 No. 1 (January 2003).

1. The Year of Cloning
Word in 2002 of new clones—including rabbits, cows, and a housecat—brings the tally of successfully cloned species to seven. The cloned cows had a human antibody gene added; the cloned pigs had a troublesome pig gene subtracted. And there was more than one boast that a human clone is in the works. How and why have we come this far?

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