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Dos ovários à clonagem e edição de genomas em modelos animais

Isadora Andre Rosa Lopes

Pelotas, 2020

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Dos ovários à clonagem e edição de genomas em modelos animais

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Para minha família, com carinho e gratidão.

Dedico.

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis."

José de Alencar

Resumo

LOPES, Isadora Andre Rosa. **Dos ovários à clonagem e edição de genomas em modelos animais.** 2020. 123f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Devido a questões éticas, é necessário testar novos fármacos em modelos animais durante a fase pré-clínica. Entretanto, existe uma necessidade emergente de novos modelos que abordem os requisitos de investigação sobre as doenças de forma mais translacional aos humanos, a qual vem sendo suprida por modelos animais editados geneticamente. A principal maneira de gerar estes animais editados, principalmente quando a espécie de escolha é a suína, é através da combinação das técnicas de clonagem e edição de genomas. Com isso, foi realizada uma revisão de literatura abordando as duas técnicas de clonagem mais utilizadas para geração de tais animais, sendo elas a transferência nuclear de células somáticas (TNCS) e a *handmade cloning* (HMC) explorando suas limitações e avanços. Etapas fundamentais como a escolha da célula doadora de núcleo e do citoplasma receptor, seguido da reconstrução do embrião clonado e transferência para uma receptora também foram exploradas. Ademais, a técnica de reclonagem utilizada para manutenção desses animais, bem como suas vantagens e desvantagens também foi abordada em uma revisão própria. Como resultado pode-se apontar uma limitação nas etapas de coleta, armazenamento e transporte de ovários. Assim, desenvolveu-se uma proposta de um recipiente capaz de manter as gônadas na temperatura correta por um maior tempo. Além disso, através da escrita de dois artigos de revisão pode-se contribuir com o conhecimento científico e aprimoramento dos processos de geração e manutenção de modelos animais geneticamente editados.

Palavras-chave: TNCS, HMC, CRISPR, Modelo animal, Clonagem.

Abstract

LOPES, Isadora Andre Rosa. **From ovaries to cloning and genome editing in animal models.** 2020. 123f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas

Due to ethical issues, it is necessary to test new drugs in animal models during the preclinical phase. However, there is an emerging need for new models that accurately model the pathology of human diseases, which has been achieved by genetically edited animal models. The main way to generate these edited animals, especially when the species of choice is swine, is through the combination of cloning and genome editing techniques. In this sense, a literature review was carried out addressing the two most used cloning techniques for the generation of such animals, namely the nuclear transfer of somatic cells (SCNT) and handmade cloning (HMC) exploring their limitations and advances. Fundamental steps such as choosing the nucleus donor cell and the recipient cytoplasm, followed by the reconstruction of the cloned embryo and transfer to a recipient were also explored. In addition, the recloning technique used to maintain these animals, as well as its advantages and disadvantages, was also addressed in an own review. As a result, a limitation in the stages of collection, storage and transport of ovaries could be pointed out. Thus, a proposal for a container capable of keeping the gonads at the correct temperature for a longer time was developed. In addition, through the writing of two review articles, it was possible to contribute with scientific knowledge and improve the processes of generation and maintenance of genetically edited animal models.

Keywords: TNCS, HMC, CRISPR, Animal model, Cloning.

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Lista de Abreviaturas

3Rs	Reduction, Refinement, Replacement
Cas	CRISPR *associated protein*
CIV	Cultivo *in vitro*
CRISPR	*Clustered Regularly Interspaced Short Palindromic Repeats*
crRNA	CRISPR RNA
DNA	*Deoxyribonucleic acid*
EUA	Estados Unidos da América
Flp	*Flippase*
FRT	*Flippase recognition site*
G2	Pós-sintética
HMC	*Handmade cloning*
MII	Metáfase II
PAM	Protospacer adjacent motif
pb	Pares de base
RH	Reparação homóloga
RNA	*Ribonucleic acid*
RNH	Reparação não homóloga
S	Sintética
sgRNA	*Single guide RNA*
TALEN	*Transcription activator-like effector nucleases*
TNCS	Transferência Nuclear de Células Somáticas
WOW	*Well-of-the-Well*
ZFN	*Zinc Finger Nuclease*

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1 INTRODUÇÃO GERAL

Devido a questões éticas, é necessário testar novos fármacos em modelos animais durante a fase pré-clínica, antes de iniciar a experimentação humana, a fim de avaliar a eficácia, toxicidade e segurança da nova terapia em questão (JUNOD, 2008). Tais modelos precisam ser semelhantes biologicamente à doença humana, bem como demonstrar resposta similar às intervenções clínicas eficazes em humanos (DENAYER; STÖHRN; VAN ROY, 2014). Assim, modelos animais têm desempenhado um papel central ao longo dos séculos em investigações científicas de doenças humanas e estratégias de tratamento, entretanto, existe uma necessidade emergente de novos modelos que abordem os requisitos de investigação sobre as doenças de forma mais translacional aos humanos (DENAYER; STÖHRN; VAN ROY, 2014).

O desenvolvimento de modelos animais editados geneticamente revolucionou a ciência, possibilitando alterações precisas em *locus* genômicos escolhidos em células, ou no animal inteiro (ZAREI et al., 2019). Tal capacidade foi estabelecida e aprimorada em uma velocidade sem precedentes, graças ao desenvolvimento da tecnologia de edição do genoma (WANG et al., 2020). A principal maneira de gerar estes animais editados, principalmente quando a espécie de escolha é a suína, é através da combinação das técnicas de clonagem e edição gênica, as quais exigem diversas etapas (SHEETS et al., 2016).

O processo de clonagem envolve duas células diferentes: o oócito e a célula doadora (CZERNIK et al., 2019). A primeira é encontrada nos ovários, e pode ser recuperada pré ou post-mortem através da aspiração dos folículos ovarianos (FORTUNE, 1994; PINCUS; ENZMANN, 1935). Vale ressaltar que os oócitos são estruturas extremamente sensíveis à temperatura, dessa forma o armazenamento e transporte correto de ovários (quando coletados post-mortem) são essenciais para a qualidade dessas células, o que é fundamental, visto que as mesmas serão posteriormente enucleadas e fornecerão os componentes celulares necessários para a reprogramação genética e o desenvolvimento embrionário inicial do clone gerado (NAOI et al., 2008).

A segunda é a célula doadora, e seu núcleo é injetado no oócito enucleado, onde será reprogramado (CZERNIK et al., 2019). No caso da geração de um modelo animal inovador, geralmente esta célula será editada (YAO; HUANG; ZHAO, 2016). A edição gênica pode ser realizada através de diversas abordagens (YAO; HUANG; ZHAO, 2016). Atualmente a técnica de CRISPR/Cas é considerada padrão ouro, possibilitando edição de genes em apenas um passo, tornando-a uma ferramenta simples, mas poderosa, para edição de genomas (JIANG; DOUDNA, 2017).

Finalmente, o oócito reconstruído (oócito + núcleo da célula doadora) é ativado, e o embrião formado pode ser utilizado tanto para pesquisa básica quanto pode ser transferido para uma receptora para desenvolver até o termo (CZERNIK et al., 2019). É importante ressaltar que a técnica irá apresentar resultados divergentes de acordo com a espécie, tipo celular do núcleo doador, qualidade do oócito, sincronização do ciclo celular, bem como o protocolo de clonagem escolhidos (CAMPBELL et al., 1996; WILMUT et al., 2002).

Ademais, após o êxito nas etapas anteriores e a geração do modelo animal de interesse, é possível manter esses animais através da técnica de reclonagem, garantindo uma cópia exata (HOLM; ALSTRUP; LUO, 2016). Tal técnica consiste na utilização de uma célula proveniente de um embrião, feto ou animal clonado, como doadora de núcleo para uma nova rodada de clonagem (WILLADSEN, 1989). Assim, como pode-se perceber, diversos avanços já foram realizados em busca do aperfeiçoamento das técnicas previamente citadas, todavia, ainda existem diversas carências que necessitam ser supridas, e esta revisão de literatura tem como objetivo apontá-las e hipotetizar alternativas para as mesmas.

2 REVISÃO BIBLIOGRÁFICA

2.1 Modelos animais

Devido a questões éticas, é necessário testar novos fármacos em modelos animais durante a fase pré-clínica antes de iniciar os testes em humanos, a fim de avaliar a eficácia, toxicidade e segurança da nova terapia em questão (JUNOD, 2008). Em relação às responsabilidades éticas envolvidas na realização de experimentos com animais, testes *in vivo* devem ser conduzidos com base no princípio dos 3 Rs, assim denominado em função das iniciais, em inglês, de seus principais objetivos: redução (*Reduction*), refinamento (*Refinement*) e substituição (*Replacement*) (BALLS, 1994; FLECKNELL, 1994; RUSSELL; BURCH, 1959; SCHECHTMAN, 2002).

Propostos inicialmente por Russel e Burch em 1959 (RUSSELL; BURCH, 1959), os 3Rs foram amplamente aceitos e adaptados à sociedade moderna em geral e à pesquisa científica (CHAPMAN et al., 2013) e, atualmente, são incorporados como um conceito-chave em diversas legislações importantes que regulamentam o uso de animais em pesquisas (FILIPECKI et al., 2011; KONG; QIN, 2010; KUROSAWA, 2007; PEREIRA et al., 2004). Dessa forma, os princípios dos 3Rs primam por estudos com animais apenas quando o objetivo for de importância justificável, não houverem métodos alternativos válidos e exista benefício científico máximo (RUSSELL; BURCH, 1959). Todas as estratégias relevantes de redução e refinamento devem ser implementadas através de um bom design experimental, minimizando assim o prejuízo causado ao bem estar animal (GUHAD, 2005; RICHMOND, 2002).

Assim, os modelos biológicos precisam ser desenvolver de forma biologicamente semelhante à doença humana em questão, demonstrando resposta similar às intervenções clínicas eficazes em humanos, e seus alvos de investigação devem possuir um papel equivalente no modelo da doença com a situação clínica de pacientes (DENAYER; STÖHRN; VAN ROY, 2014). Sendo assim, os animais devem ser capazes de mimetizar de forma confiável a anatomia e a fisiologia normal dos órgãos e tecidos humanos de interesse, além de refletir com precisão os aspectos morfológicos e bioquímicos da patogênese da doença (DENAYER; STÖHRN; VAN ROY, 2014). No entanto, é comum enfrentar uma dificuldade na tradução da resposta obtida entre o animal pré-clínico e os humanos (MAK; EVANIEW; GHERT, 2014).

Ao longo dos tempos, tem-se verificado um aumento do número de terapêuticas conduzidas em fase de ensaio clínico. Porém, a maior parte dos ensaios de fase III falham no alcance dos seus *endpoints* primários. Nos EUA, mais de 30% dos medicamentos promissores falharam em testes clínicos porque foram considerados prejudiciais à saúde humana (NATIONAL INSTITUTE OF HEALTH, 2020). Surge assim, a necessidade de melhorar a predição dos modelos animais utilizados na avaliação terapêutica, uma vez que os mesmos são cruciais para a validação e descoberta de novos medicamentos.

Os roedores têm sido historicamente a plataforma mais utilizada para triagem pré-clínica e como modelos biológicos em geral (POLEJAEVA; RUTIGLIANO; WELLS, 2016). Algumas de suas características como o seu pequeno tamanho, o baixo custo, e a genética bem conhecida, os tornam uma ferramenta padrão para avaliar novas terapêuticas (VANDAMME, 2014). Sem mencionar o fato de que eles podem ser geneticamente modificados com bastante facilidade (ZAREI et al., 2019). No entanto, eles nem sempre modelam com precisão a patologia de doenças humanas (BURNS et al., 2015; JUNHEE SEOK et al., 2013).

Por outro lado, os suínos já provaram serem mais preditivos de tratamentos terapêuticos em seres humanos do que roedores (MEURENS et al., 2012), fornecendo uma plataforma ideal devido às suas semelhanças com humanos nos níveis anatômico, fisiológico, metabólico e genético (SCHOOK et al., 2015). Ademais, como animais de produção, há ampla aceitação pública de seu uso, o que não é o caso de outras espécies não-roedoras, como os primatas. Outro fator importante é que o Comitê Internacional de Harmonização exige que testes de toxicidade sejam realizados em pelo menos duas espécies animais relevantes a fim de comprovar a eficácia da terapia antes de iniciar os ensaios clínicos em humanos (FOOD AND DRUG ADMINISTRATION, 2008). Dessa forma, o modelo suíno se apresenta como excelente candidato, uma vez que quando utilizado em testes posteriores aos realizados em modelos animais de pequeno porte (roedores) possibilita resultados mais fidedignos e translacionais aos humanos (SEGATTO et al., 2017) .

A sequência de genoma do suíno publicada em 2012 forneceu informações importantes sobre a sua semelhança genética com os seres humanos (GROENEN et al., 2012), bem como ajudou a consolidar sua aceitação como um modelo biomédico

de grande porte para doenças humanas (PRATHER et al., 2013; SCHOOK et al., 2015). Atualmente, suínos transgênicos estão sendo cada vez mais aceitos e utilizados em estudos de diversas doenças humanas (AIGNER et al., 2010), visto que já foram estabelecidos para doenças neurodegenerativas (KRAGH et al., 2009), fibrose cística (ROGERS et al., 2008), doenças cardiovasculares (HAO et al., 2006), diabetes mellitus (RENNER et al., 2010) e até mesmo câncer (FLISIKOWSKA et al., 2012; SCHOOK et al., 2015).

2.2 Edição gênica

Geralmente, as tecnologias aplicadas à engenharia genética podem ser divididas naquelas mediadas em embriões, e nas mediadas em células. A primeira estratégia aborda a modificação genética diretamente em embriões em estágios de pré-implantação (WHEELER, 2003; WHEELER; WALTERS, 2001). Já no segundo caso, a informação genética é introduzida em células em cultivo, sendo elas células tronco, somáticas ou gametas. Independentemente do protocolo de edição escolhido, um animal pode ser gerado, sendo através da transferência do embrião editado ou ainda utilizando a técnica de clonagem seguida da transferência do embrião clonado (SATO et al., 2016).

Modificações genéticas, incluindo a moderna estratégia de edição gênica CRISPR/Cas, quando realizadas em cultivo celular devem ser procedidas pela seleção de colônias de células que sofreram a modificação genética desejada (Figura 1) (BERTOLINI et al., 2016). Tal modificação pode ser uma deleção de sequências genômicas, ou ainda uma integração do DNA de interesse no genoma hospedeiro (GALLI et al., 2012). Este processo é seguido pela triagem molecular das colônias selecionadas, o que permite a determinação do genótipo e possíveis polimorfismos, do número de cópias e da localização cromossômica do DNA exógeno no genoma do hospedeiro (Figura 1) (KONG et al., 2014). Além disso, a capacidade de utilizar uma população clonal de cultivos transgênicos garante o mesmo local de inserto em todos os clones, diminuindo assim a variação de animal para animal nos níveis de expressão de um transgene, além de possibilitar armazenamento por longos períodos de tempo através do congelamento da linhagem (CIBELLI et al., 2013).

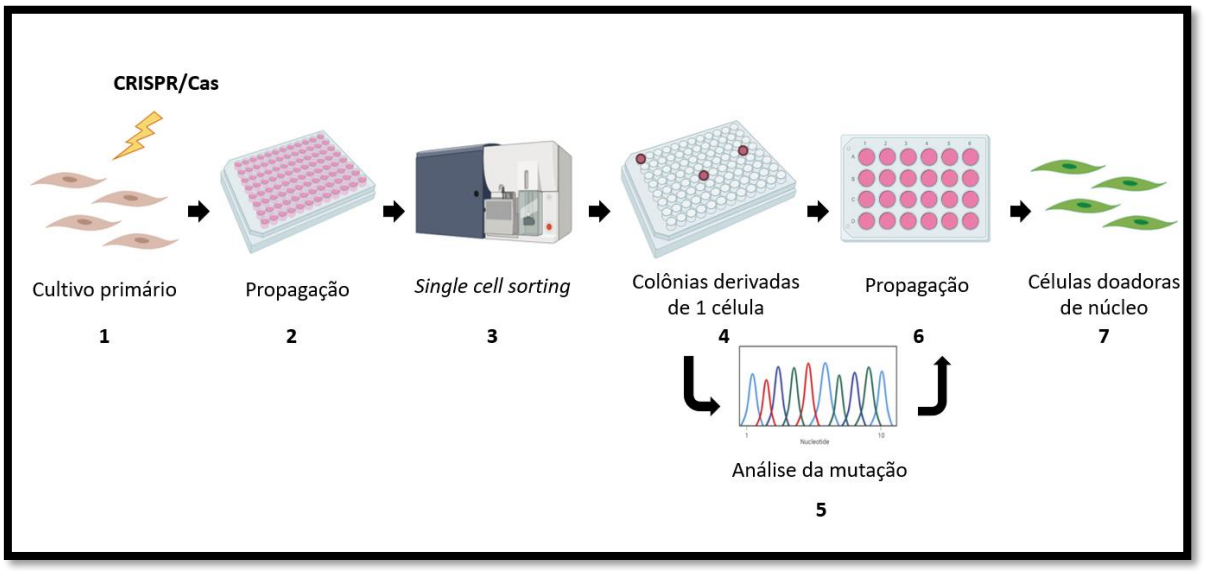

Figura 1. Representação esquemática do isolamento e triagem molecular de células editadas para utilização na técnica de clonagem. Primeiramente o cultivo primário é submetido à edição gênica (1), após deve ser cultivado sob condições adequadas (2) para que possa se realizar a técnica de "cell sorting" (3) resultando na seleção e separação unicelular das células que contém a modificação (4). Posteriormente, a análise da mutação presente no genoma com sequenciamento de Sanger será realizada e apenas as colônias que contém a mutação desejada devem ser propagadas (6) para então serem utilizadas como células doadoras de núcleo (7). Fonte: Figura criada com BioRender.

2.2.1 Nucleases

Após as maiores revoluções científicas do século XX, como o desdobramento da estrutura do DNA (CRICK; WATSON, 1953), do código genético (CRICK, 1966) e da tecnologia do DNA recombinante (COHEN et al., 1973), grandes descobertas já foram realizadas no século XXI. Um importante exemplo são as nucleases engenheiradas utilizadas na edição de genomas, tais como ZFN (do inglês, *zinc finger nuclease*), TALEN (do inglês, *transcriptionactivator-like effector nuclease*) e o sistema CRISPR/Cas9 (do inglês, *clustered regularly interspaced short palindromic repeats/associated system*) (DOUDNA; CHARPENTIER, 2014; JABALAMELI et al., 2015; MILLER et al., 2011).

As ZFNs e as TALENs são proteínas quiméricas projetadas para reconhecer e clivar sequências específicas de nucleotídeos no DNA genômico, assim possibilitando a realização de alterações sítio dirigida (DURAI et al., 2005; SUN; ZHAO, 2013; WOOD et al., 2011). As duas tecnologias são muito similares, pois utilizam o mesmo domínio de corte do DNA, denominado FokI (JOUNG; SANDER, 2013). No entanto, essas técnicas apresentam como principais limitações a necessidade de desenhar,

sintetizar e validar as proteínas “engenheiradas”, o que as tornam inviáveis para o uso rotineiro (GUPTA; MUSUNURU, 2014).

Como alternativa, foi desenvolvida a tecnologia CRISPR/Cas, que tem se mostrado mais eficiente quando comparado às técnicas ZFN e TALENS (KENNEDY; CULLEN, 2015). O sistema CRISPR é um sistema imune adaptativo utilizado por bactérias para proteção contra vírus invasores e plasmídeos baseado na clivagem sequencial de ácidos nucléicos estranhos à célula (DOUDNA; CHARPENTIER, 2014; HSU et al., 2013). Recentemente, tal sistema foi adaptado à edição de genomas através da geração do sistema CRISPR/Cas, ferramenta alternativa que vem sendo considerada de grande eficiência, além de possuir simplicidade de criar o direcionamento, baixo custo, e levar um curto espaço de tempo de montagem (CONG; ZHANG, 2015; DOUDNA; CHARPENTIER, 2014).

Atualmente o sistema mais adequado para a maioria das aplicações na engenharia genética é o sistema CRISPR Cas Tipo II (derivado de *Streptococcus pyogenes*), o qual utiliza uma proteína conhecida por Cas9 (SANDER; JOUNG, 2014). O processo baseia-se na inserção de um RNA guia único (sgRNA) de 20 nucleotídeos complementar à uma sequência de DNA alvo (CONG et al., 2013; JINEK et al., 2012). Para a Cas 9 se ligar com sucesso ao DNA, tal sequência alvo deve ser precedida por uma sequência chamada protospacer motifs adjacentes (PAM) (SANDER; JOUNG, 2014). Assim, após realizada a ligação, ocorre a quebra de dupla fita no local de interesse (CONG et al., 2013; JINEK et al., 2012).

Essas três metodologias citadas anteriormente possibilitam a geração de quebras duplas em locais específicos da molécula de DNA, que são reparadas por dois principais mecanismos: a reparação homóloga (RH) e a reparação não homóloga (RNH) (CECCALDI; RONDINELLI; D’ANDREA, 2016). Estes dois sistemas de reparo diferem em sua necessidade de um DNA modelo homólogo e na fidelidade do reparo da quebra de dupla fita. O reparo dirigido pela RH é, em grande parte, um mecanismo livre de erros, pois utiliza como modelo a informação genética contida na cromátide-irmã não danificada, ou no caso da edição gênica, utiliza o DNA inserido como modelo (LI; HEYER, 2008). Em contraste, a RNH é normalmente propensa a erros, e promove a eliminação da quebra de fita dupla pela ligação direta das extremidades quebradas, podendo remover ou adicionar bases, causando mutações (LIEBER, 2011). A RNH é

a via de reparo mais frequente nas células de mamíferos operando em todas as fases do ciclo celular, enquanto a RH é restrita às fases tardia S e G2 (CECCALDI; RONDINELLI; D'ANDREA, 2016).

2.2.2 Sistemas de recombinação

As recombinases de DNA são amplamente utilizadas para manipular a estrutura do genoma e controlar a expressão gênica (NERN et al., 2011). Os sistemas de recombinação mais utilizados em animais atualmente incluem o sistema Flp-FRT e o sistema cre-loxP. O primeiro já foi intensamente estudado e sua eficácia foi demonstrada em uma ampla gama de organismos (ANAND et al., 2019; BOWDEN; PALANI; LIBOUREL, 2017; MEI et al., 2019; PERRY; BELLO; SMITH, 2020). Tal sistema de recombinação é mediado pela recombinase Flp (flippase), derivada de *Saccharomyces cerevisiae* (ZHU; SADOWSKI, 1995), e os locais do alvo de reconhecimento que devem flanquear o gene de interesse possuem 34pb e são denominados FRT (derivado do inglês - *flippase recognition target*). Assim, quando a recombinase Flp é expressa, o local do gene flanqueado diretamente pelos locais FRT é eliminado do genoma (ZHU; SADOWSKI, 1995).

Já o segundo é o sistema de recombinação mediado por cre-loxP (o "sistema cre-loxP"), uma poderosa ferramenta de edição de genes que se tornou extremamente importante e amplamente utilizada para a pesquisa em genética e biologia celular. Sternberg e Hamilton (1981) foram os primeiros a descrever uma enzima recombinase isolada do bacteriófago P1 que recombina fragmentos de DNA, a qual foi nomeada "cre" por causar recombinação, e chamaram seus locais de ligação "LoxP" advindo de lócus de cruzamento de (x), P1 (STERNBERG; HAMILTON, 1981). Tal sistema permitiu a análise de funções genéticas específicas em linhagens celulares de uma ampla gama de tecidos e contextos fisiológicos, culminando no avanço fundamental de nosso conhecimento biológico.

A operação do sistema cre-loxP depende de dois principais elementos, primeiramente uma linhagem celular ou animal deve ser projetada para transportar um locus genético no qual o DNA será flanqueado por dois motifs de DNA de 34-pb loxP (SAUER; HENDERSON, 1988). Posteriormente, a enzima cre deve ser expressa em uma célula contendo DNA floxado. Assim, a especificidade celular da

recombinação é controlada por sequências promotoras que dirigem a expressão cre no tipo de célula ou de interesse (GU; ZOU; RAJEWSKY, 1993), eliminando assim o locus genético floxado apenas no local de interesse.

Uma grande inovação em relação à tecnologia cre-loxP tem sido o desenvolvimento de controle temporal e celular específico da atividade da cre, permitindo a indução experimental da recombinação do DNA (SAUER, 1998). Para tal fim, o sistema cre-loxP sofreu modificações para que a localização intranuclear de cre ou a expressão de cre possam ser reguladas. Sistemas induzíveis permitem o estudo de genes ou células em que a exclusão global ou embrionária dos lócus floxados pode ser prejudicial, sendo esse o caso quando genes de interesse são essenciais para o desenvolvimento.

2.3 Métodos de clonagem

Atualmente, duas técnicas têm sido utilizadas em suínos para a produção de embriões clonados. A primeira é o método tradicional de clonagem, também conhecido como Transferência Nuclear de Células Somáticas (TNCS), e a outra é a clonagem manual (handmade cloning-HMC).

2.3.1 Transferência Nuclear de Células Somáticas

A Transferência Nuclear de células Somáticas é utilizada na maioria dos laboratórios que realizam clonagem em mamíferos. Tal técnica envolve a remoção dos cromossomos haplóides (1n) de um oócito em estágio de metáfase II (MII) etapa conhecida como enucleação (ROSS; FELTRIN, 2014). Posteriormente, ocorre a transferência e fusão de uma célula somática diplóide (2n) em um oócito previamente enucleado (ROSS; FELTRIN, 2014). Finalmente, o oócito reconstruído é então ativado artificialmente por meio de pulsos elétricos ou estimulação química, induzindo o desenvolvimento do embrião, que será posteriormente transferido para uma receptora, onde irá atingir o desenvolvimento completo (Figura 2) (ROSS; FELTRIN, 2014).

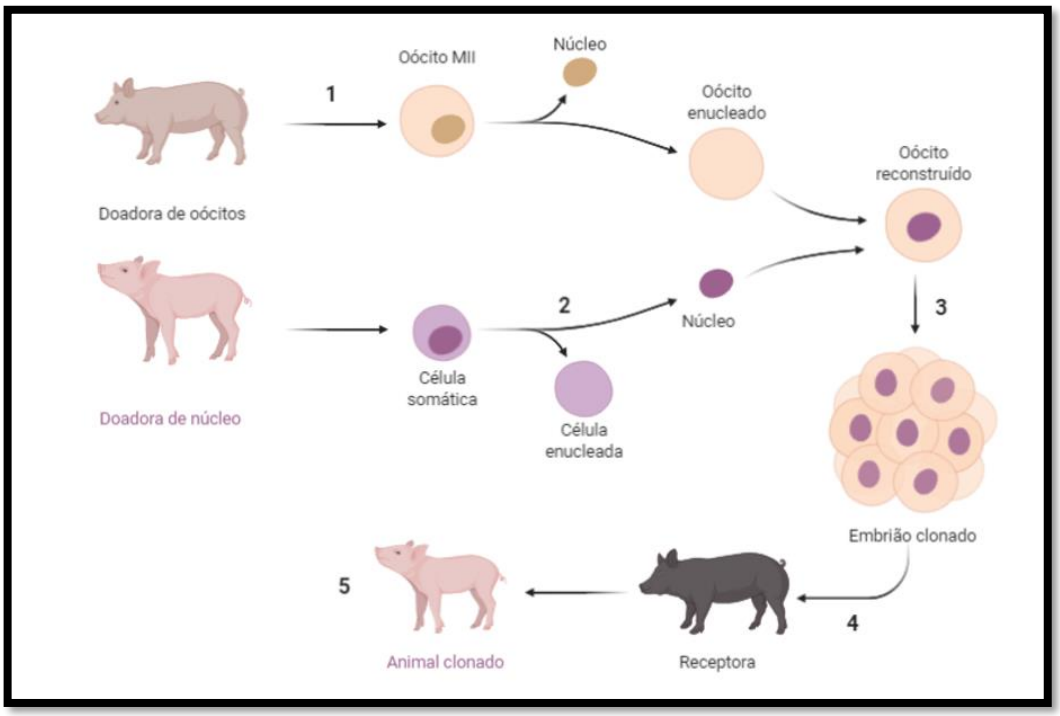

Figura 2. Representação esquemática do procedimento para realização da técnica TNCS. 1. Oócitos são coletados de ovários provenientes de abatedouro ou em porcas vivas, enucleados e utilizados como células receptoras. 2. Fibroblastos são coletados através de biopsia de porcos vivos ou fetos e em seguida submetidos a cultivo primário para utilização como célula doadora de núcleo. 3. O núcleo doador é inserido no oócito enucleado. 4. Após a ativação o embrião será cultivado in vitro até o estágio desejado e transferido para uma receptora sincronizada. 5. Após a geração do animal clonado a técnica de reclonagem pode ser utilizada para manter o modelo criado. Fonte: Figura criada com BioRender.

A primeira progênie a ser criada por TNCS utilizando uma célula somática adulta foi a ovelha Dolly (WILMUT et al., 1997). Em seguida, diversos mamíferos incluindo suínos (BETTHAUSER et al., 2000), bovinos (KATO et al., 1998; LUO et al., 2015), camundongos (TANABE et al., 2017; WAKAYAMA et al., 1998), cabras (FENG et al., 2015), gatos (SHIN et al., 2002), e primatas não humano (LIU et al., 2019) foram clonados com sucesso. Além disso, quando células editadas geneticamente são utilizadas como doadoras, diversos modelos animais humanizados podem ser produzidos, como já demonstrado em suínos (HUANG et al., 2017; YAN et al., 2018; YU et al., 2018; ZHOU et al., 2014).

Apesar das conquistas anteriores, a eficiência geral da clonagem permanece baixa (TAN et al., 2016). Os principais fatores que dificultam o sucesso da técnica incluem a necessidade de equipamentos de alto custo e de pessoal habilitado para operar os mesmos, bem como a reprogramação inadequada do núcleo doador. Além disso, mesmo quando a técnica é bem-sucedida e o embrião clonado produzido, o

mesmo ainda pode sofrer de diversos defeitos de desenvolvimento que muitas vezes serão incompatíveis com a vida (SCHMIDT et al., 2015), o que acaba reduzindo ainda mais a eficiência.

2.3.2 *Handmade cloning*

Handmade cloning (HMC), também conhecida como clonagem manual, é uma técnica que supre as necessidades de utilizar equipamentos sofisticados (micromanipuladores) e de possuir pessoal altamente qualificado (VAJTA et al., 2000). O principal diferencial da técnica é que durante o procedimento, os oócitos devem estar livres da zona pelúcida, como demonstrado na figura 3 (VAJTA et al., 2000). Na HMC, a enucleação dos oócitos maturados ocorre através bissecção com auxílio de microlâminas e sob um estereomicroscópio, para obtenção de hemi-citoplastos (VERMA et al., 2015). Subsequentemente, ocorre a aproximação de dois hemi-citoplastos ao núcleo doador para que o processo de fusão aconteça, este podendo ser realizado em uma ou duas etapas, através de pulsos elétricos (VAJTA, 2007). Finalmente, o oócito recontruído deverá ser submetido à ativação química para formação do embrião clonado (VAJTA, 2007; VERMA et al., 2015).

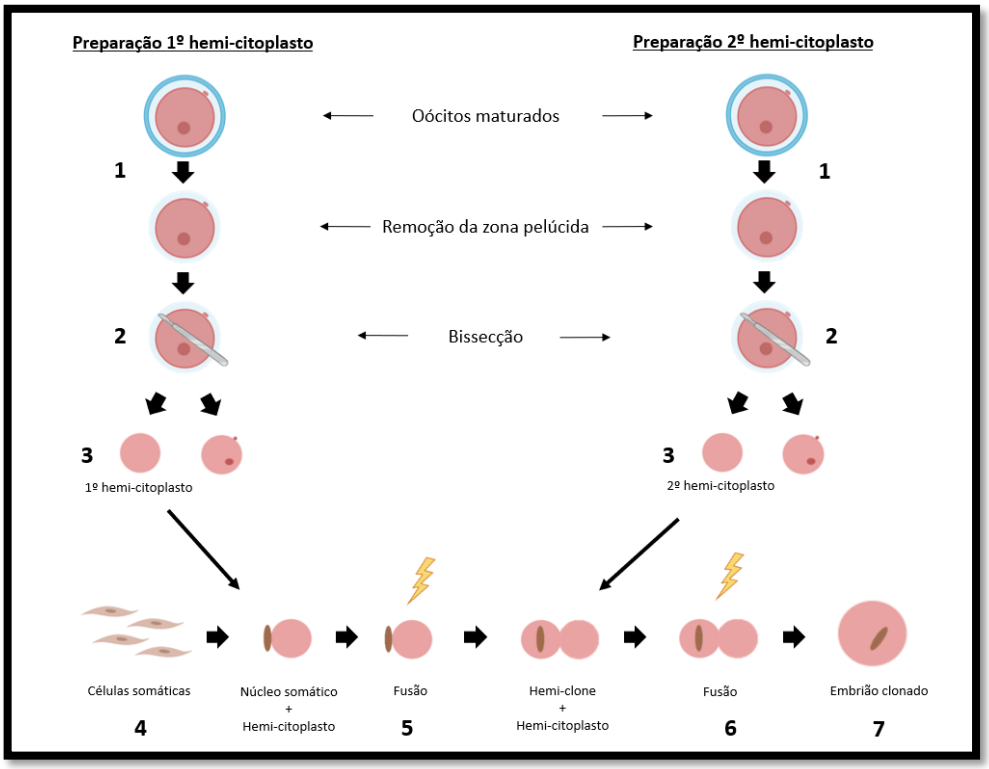

Figura 3. Representação esquemática do procedimento para realização da técnica HMC. Primeiramente ocorre a preparação dos hemi-citoplastos, na qual oócitos passarão pela remoção da zona pelúcida (1), e bissecção com micro lâmina (2), sendo separadas para o uso apenas as porções sem DNA (3). Em seguida, células somáticas serão utilizadas como doadoras de núcleo (4), o qual será fusionado a um hemi-citoplasto (5), formando um hemi-clone, que será fusionado com o segundo hemi-citoplasto (6) para a formação do embrião clonado (7). Fonte: Figura criada com BioRender.

Posteriormente, o sistema de cultivo de embriões sem zona pelúcida (ZP), denominado Well-of-the-Well, ou WOW deve ser utilizado para cultivar com eficiência embriões individuais até o estágio de blastocisto e evitar agregação (VAJTA et al., 2000). Conjuntamente com esse sistema, meios com elevado conteúdo de macromoléculas (VAJTA, 2007), ou até mesmo uma zona pelúcida artificial também podem ser implementados (US Patent No. 5272086A, 1993). Por conseguinte, os blastocistos devem ser transferidos para receptoras sincronizadas.

A HMC tem sido utilizada em diversos animais de fazenda, incluindo búfalo (PANDA et al., 2011), ovelha (ZHANG et al., 2013), cavalo (LAGUTINA et al., 2007) e porco (DU et al., 2007). Possuindo taxas de desenvolvimento comparáveis ou até maiores do que a técnica de clonagem convencional na espécie suína (LAGUTINA et al., 2007; ZHANG et al., 2012). As principais vantagens da técnica incluem o procedimento simples e rápido e a possibilidade da universalização do acesso à técnica de clonagem a todos os pesquisadores, laboratórios e institutos de pesquisa,

incluindo aqueles com menor disponibilidade de recursos para aquisição de equipamentos. Já as desvantagens incluem a heteroplasmia mitocondrial decorrente da utilização de citoplasmas provenientes de dois oócitos diferentes, bem como um aumento na necessidade de oócitos de qualidade (VAJTA, 2007).

2.4 Célula receptora

A célula receptora é responsável pela reprogramação do núcleo doador, uma vez que desempenha um papel crítico na ativação e subsequente desenvolvimento dos embriões clonados (LORTHONGPANICH; SOLTER; LIM, 2011; SINGH et al., 2019). Dessa forma, a escolha de tal célula é fundamental para o sucesso da clonagem, e atualmente oócitos em metáfase II (MII) têm sido a principal escolha na espécie suína, por conterem todos os fatores necessários para reprogramar de forma eficiente os núcleos diferenciados das células somáticas (LORTHONGPANICH; SOLTER; LIM, 2011).

A recuperação desses oócitos é realizada através da punção folicular ovariana, podendo ser *in vivo*, utilizando fêmeas estimuladas por hormônios, ou *post-mortem*, utilizando ovários provenientes de abatedouros (FORTUNE, 1994; PINCUS; ENZMANN, 1935). Atualmente, sabe-se que, principalmente para pesquisa, a aspiração post-mortem é a mais utilizada, devido a sua ampla disponibilidade e baixo custo. Entretanto, a distância entre o local do abatedouro e o laboratório de pesquisa pode se tornar um empecilho para manutenção da qualidade dessas gônadas, e, consequentemente dos oócitos.

A qualidade do oócito é um fator limitante para o sucesso da técnica de clonagem. Todavia, já foi descrito que tais estruturas são extremamente sensíveis a mudanças de temperatura e processos de hipóxia, ambos comumente decorrentes do longo tempo de transporte em recipientes inadequados (NAKAO; NAKATSUJI, 1992). De fato, na espécie suína, um curto espaço de tempo e temperatura estável parecem ser as condições de transporte mais adequadas dos ovários para a manutenção da qualidade oocitária. Mais precisamente, o armazenamento ideal varia de 25-35ºC por 2-3 horas (TELLADO et al., 2014; WONGSRIKEAO et al., 2005). Já quando utilizada uma temperatura baixa (15ºC), ou períodos superiores a 6 horas, tanto a maturação

oocitária quanto as taxas de desenvolvimento embrionário diminuem (LIN et al., 2011; WONGSRIKEAO et al., 2005).

Geralmente, o transporte e armazenamento de ovários coletados em abatedouros são realizados em garrafas térmicas comuns disponíveis no mercado. Tal recipiente é inadequado, uma vez que no decorrer da coleta acabam ficando abertos, expondo de forma prolongada os ovários ao ambiente, gerando trocas de temperaturas e consequente perda de calor presente nos ovários. Além disso, mesmo depois de fechadas, essas acabam preservando pouco a temperatura inicial dos ovários, o que pode acabar prejudicando os resultados finais de desenvolvimento embrionário.

2.5 Célula doadora de núcleo

A seleção da célula doadora de núcleo é outro aspecto fundamental para alcançar uma eficiente reprogramação nuclear e clonagem (CZERNIK et al., 2019; YOO et al., 2017). Com isso, diversas publicações observando e comparando o efeito do tipo de célula, estágio do ciclo celular e consequência na qualidade da produção de embriões foram publicadas (LI et al., 2013; WEI et al., 2013). Mais precisamente na espécie suína, os fibroblastos (fetais e adultos) são a principal escolha para tipo de célula doadora, uma vez que apresentam fácil amostragem, isolamento e manutenção *in vitro* (SINGH et al., 2019; YANG et al., 2016). Tal manutenção possibilita a modificação genética e seleção da população de células com a modificação de interesse antes da reconstrução do embrião e da geração do modelo animal, constituindo assim uma grande vantagem.

Os fibroblastos fetais já foram relatados como uma melhor opção quando comparados com adultos, por apresentarem maior eficiência de animais nascidos, bem como menor índice de anormalidades de desenvolvimento (LIU et al., 2015). Entretanto, a escolha de utilizar uma célula derivada de um feto limita o conhecimento do fenótipo do futuro animal, que muitas vezes poderia possuir alguma anormalidade, influenciando no sucesso da técnica de clonagem. Assim, eleger um fibroblasto adulto com o fenótipo conhecido e sem possuir anormalidades pode ser uma melhor abordagem.

Após a escolha do tipo celular, outros fatores ainda devem ser levados em consideração, como o número de passagens dessas células, já que segundo Li e colaboradores (2014) células com mais de oito passagens impossibilitam a formação de prole, indicando o uso de linhagens com menos de seis passagens (LI et al., 2014). Além disso, o sexo e raça do animal que a célula doadora será isolada, assim como a presença de determinadas modificações genéticas também podem influenciar o resultado da técnica (SCHMIDT et al., 2015). Por fim, a coordenação simultânea entre o ciclo celular do núcleo do doador e o oócito receptor também é de extrema importância para reprogramação celular e eficiência na clonagem (CAMPBELL et al., 1996; WELLS et al., 2003).

Para uma correta reprogramação quando oócitos MII são utilizados como citoplasmas receptores, estudos apontam que a célula doadora deve estar em estágio quiescente (G0) ou parada na fase G1 do ciclo celular (CAMPBELL et al., 1996; KUES et al., 2000). Diferentes protocolos para sincronização em tais estágios estão disponíveis, todavia a inibição por contato sob condições de confluência total parece ser o método mais frequentemente utilizado e menos estressante. Tal método consiste em cultivar células por aproximadamente quatro dias, ou até que uma alta confluência (90%) seja atingida, resultando também em melhores taxas de prenhez, e eficiência geral de clonagem quando comparada com confluências mais baixas (JIN et al., 2018).

Além disso, no procedimento de clonagem uma diferenciação reversa conhecida como desdiferenciação é necessária para redirecionar as células somáticas para um estágio embrionário totipotente (KANG et al., 2001; MEISSNER; JAENISCH, 2006). Para isso, a cromatina proveniente da célula doadora de núcleo passa por diversas alterações epigenéticas, incluindo a inativação do cromossomo X, acetilação de histonas, metilação de DNA, bem como remodelação de proteínas associadas à cromatina (HUAN et al., 2015; IUSO et al., 2015; RUAN et al., 2018; YAMANAKA et al., 2009). Tais eventos são fundamentais para uma reprogramação eficiente, e quando não realizados corretamente podem gerar anormalidades muitas vezes incompatíveis com a vida.

2.6 Transferência de embriões

Após a ativação e cultivo *in vitro* (CIV) dos embriões clonados os mesmos deverão ser transferidos a uma receptora. A sincronização prévia do estro pode ser realizada de forma natural ou induzida quimicamente, sendo a última mais eficaz em garantir a prenhez (JIN et al., 2018; PETERSEN et al., 2008). Normalmente, a prostaglandina e combinações de prostaglandina e gonadotrofinas são utilizadas para tal indução (SINGH et al., 2019).

O protocolo de transferência de embriões irá variar de acordo com a técnica de clonagem utilizada (TNCS ou HMC), sendo que existem dois mais utilizados na espécie suína. O primeiro consiste em transferir o embrião clonado após 1 ou 2 dias de CIV, já o segundo baseia-se na transferência de embriões no estágio de blastocisto (5 dias após ativação) (JIN et al., 2018; YOO et al., 2017). Ambas as técnicas possuem suas peculiaridades e apresentam resultados satisfatórios.

Além disso, devido ao fato de os embriões suínos clonados ainda possuírem baixa capacidade de desenvolvimento, realiza-se a transferência de no mínimo 100 embriões por receptora com intuito de compensar tal limitação e obter prenhez (BETTHAUSER et al., 2000; DAI et al., 2002; KAWAKAMI et al., 2003). Logo, após uma seleção natural *in vivo* dos embriões mais competentes, geralmente restam por volta de quatro, que serão mantidos durante todo o período gestacional (BAZER et al., 1969; POLGE; ROWSON; CHANG, 1966). Outra peculiaridade da espécie suína é a escolha da raça do embrião clonado e da receptora, uma vez que principalmente quando porcos em miniatura estão envolvidos, a eficiência da clonagem pode ser influenciada devido à diferença de tamanho de útero e peso ao nascer em relação a porcos domésticos (JIN et al., 2018).

2.7 Reclonagem

Por conseguinte, as técnicas de clonagem ainda possuem diversas limitações que acarretam na baixa taxa de produção de descendentes viáveis. Além disso, grande parte das anormalidades encontradas já foram relacionadas com falhas na reprogramação celular. Assim, Willadsen e colaboladores (1989), sugeriram a utilização de um método inovador denominado reclonagem, agora também conhecido como clonagem nuclear em série, como forma de possibilitar uma maior exposição da

cromatina a fatores epigenéticos, e assim auxiliar para que o processo de reprogramação ocorra de forma correta (KUROME et al., 2008; WILLADSEN, 1989; ZAKHARTCHENKO et al., 2001).

A técnica consiste na utilização de uma célula proveniente de um embrião, feto ou animal previamente clonado como doadora para uma nova rodada de clonagem, como demonstrado na figura 4 (WILLADSEN, 1989). A técnica possui diversas aplicações potenciais, dentre as quais destacam-se a reclonagem de animais geneticamente editados vivos ou post-mortem, possibilitando assim a ressurreição e manutenção de modelos de doenças huamanas (HOLM; ALSTRUP; LUO, 2016). Além disso, já foi relatado que animais clonados e reclonados são produzidos com eficiências comparáveis (CHO et al., 2007; WAKAYAMA et al., 1998), e mais especificamente na espécie suína, resultados encorajadores em relação à produção de suínos reclonados vem sendo publicados (AHN et al., 2011; CAO et al., 2012; CHO et al., 2007).

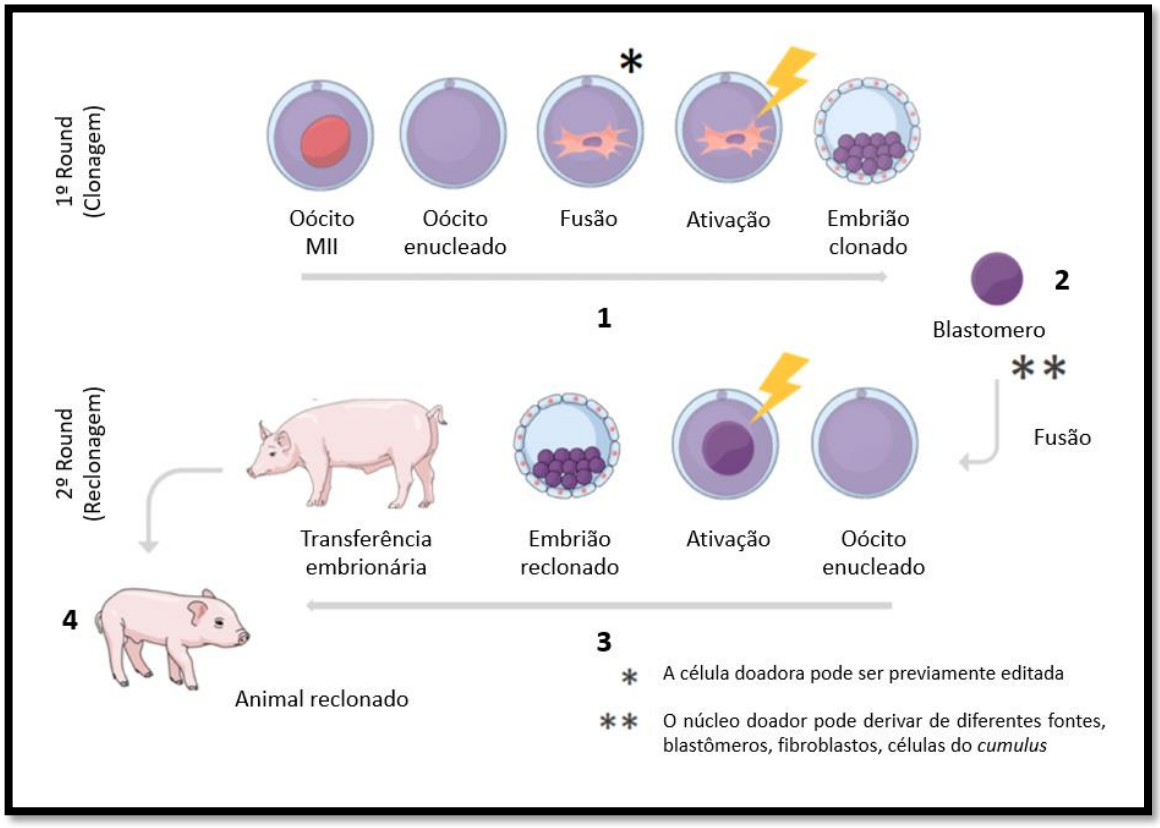

Figura 4. Representação esquemática do procedimento para realização da técnica de reclonagem. Primeiramente o processo normal de clonagem deve ser realizado (1), e posteriormente uma célula proveniente de um embrião, feto ou animal clonado (2), é utilizada como doadora para um novo processo de clonagem (3), gerando por fim o animal reclonado (4). Fonte: Figura criada com BioRender.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A produção de clones animais geneticamente editados possui limitações que podem ser supridas através de produtos ou processos inovadores.

3.2 Objetivo Geral

Elucidar sobre os principais avanços e limitações nas etapas de produção de clones animais geneticamente editados, com intuito de desenvolver uma estratégia para melhorar as taxas de desenvolvimento embrionário, resultando em prole viva.

3.3 Objetivos Específicos

- Elucidar sobre a importância da seleção dos oócitos no resultado de uma clonagem;
- Abordar sobre a importância da escolha do tipo e da linhagem celular da célula doadora de núcleo em técnicas de clonagem;
- Listar os principais protocolos de clonagem de suínos atualmente utilizados;
- Mencionar sobre as principais estratégias de reprogramação nuclear;
- Abordar sobre os protocolos de transferência embrionária utilizados na espécie suína;
- Relatar sobre a estratégia de reclonagem;
- Apontar as principais alternativas para suprir as limitações das técnicas envolvidas na clonagem;
- Desenvolver um recipiente para coleta, armazenamento e transporte de gônadas de mamíferos.

4 CAPÍTULOS

4.1 Artigo 1 – From collecting ovaries to creating an innovative pig model: A state of the art review

Artigo formatado nas normas da revista *Animal Biotechnology*

From collecting ovaries to creating an innovative pig model: A state of the art review

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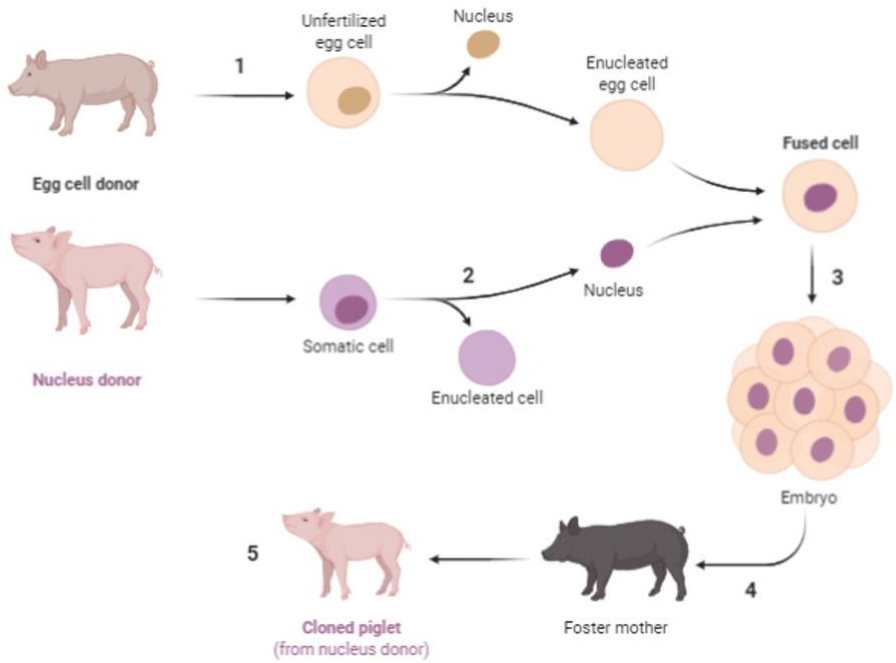
From collecting ovaries to creating an innovative pig model: A state of the art review

Due to ethical issues, it is necessary that new developed drugs are tested in animal models during the preclinical phase, prior to human testing. However, there is an emerging need for organisms that accurately model the pathology of human diseases, which has been achieved by genetically edited animal models. The main way to generate these innovative animals, especially when the species of choice is swine, is through the combination of cloning and gene editing techniques. Thus, a literature review was carried out addressing the two most used cloning techniques for the generation of such animals, namely the somatic cells nuclear transfer (SCNT) and handmade cloning (HMC), exploring their limitations and advances. Fundamental steps such as choosing the donor nucleus and the recipient cytoplasm, followed by the reconstruction of the cloned embryo and transfer to a recipient, as well as the use of recloning technique for the animal model maintenance were also explored.

Keywords: TNCS; HMC; CRISPR; Animal model; Cloning

Graphical abstract:



1 → Thermal container
MII oocytes

2 → High confluence
Low passages
Prm1

3 → Xist knockout
HDACis treatment
Dnmt1 knockdown
Kdm4 mRNA

4 → Estrus synchronization
Embryo/recipient breed
>100 embryos transeferred

5 → Recloning

1 Introduction

The Nuremberg code states that any experimentation on humans must be preceded by animal experimentation [1]. Therefore, animal models have historically played a critical role in biomedical research and drug development, and are essential to bridge the translational gap between preclinical and clinical research [2,3]. In this sense, a model is a simple representation of a complex system, and should possess similar genetic basis, anatomy, physiology, pathological response and underlying mechanism when compared to humans [2,4].

Over the years, biomedical research has especially focused on mouse models due to its known genome sequence data, small size and significant low cost [5,6]. However, they do not always accurately model the pathology of human diseases, since the rates of successful human trials have been disappointingly low [7,8]. According to the National Institutes of Health [9], more than 30 percent of promising medications have failed in clinical trials because they are found to be harmful to human health, raising concern over translation. In order to improve prediction of the clinical situation, careful selection of the species, complexity and chronicity of the models is vitally important [5].

Livestock models, such as pigs, have already proven to be more predictive of therapeutic treatments than murines [10], providing an ideal platform for biomedical research and drug development [11]. In addition, the fast breeding period, large litter size and short generation interval of pigs hold obvious advantages over small mammals [12]. However, when compared with other models, domestic farm pigs are much larger, weighing over 300 kg in adult size, hence, demanding more space and feed [11]. Minipigs, on the other hand, reach around 20-100 kg, depending on the breed, offering lower operational costs and overcoming the space issue [13].

Applications of pigs in biomedical research are abundant, particularly in the field of translational research, enabling the study of heart conditions [14–16], wound healing [17],

pharmacokinetics [18,19], gastrointestinal diseases [20], vaccine development [10], as well as different types of cancer [21,22]. Furthermore, recent advances in genome editing technology in addition to available genome sequence data allowed the researchers to make precise mutations [23]. This combined technologies helped to improve the translation between models and humans, and resulted in the creation of pig models for Laron syndrome [24], Huntington's disease [12], Parkinson's disease [25], as well as cardiovascular disease [26].

Genome editing with site-specific nucleases significantly improved efficiency of targeted mutation by providing the ability to readily disrupt genes and introduce specific mutations [27]. This technology is based on the ability of engineered nucleases to introduce a double-stranded break in a targeted position in the genome, which will stimulate either homologous recombination (HR) or nonhomologous end-joining (NHEJ) repair pathways [27]. There are two main strategies in order to develop genome edited (GE) models, more specifically, the *ex vivo*, which is genome editing at the cellular level and performed outside an organism, and the *in vivo*, which is genome editing at the organismal level [23]. For the purpose of this review, only the *ex vivo* approach will be addressed, which in combination with cloning procedures such as somatic cell nuclear transfer (SCNT) or hand-made cloning (HMC), permit the creation of animal models with desired modifications, such as humanization or insertion/deletion of genic fragments which will closely mimic a human disease.

According to the concept of Replacement, Reduction and Refinement (3Rs), the trend is to use a lower number of more sophisticated models in research [28]. However, despite its widespread use, obtaining relevant GE models still faces countless theoretical and technical challenges [29]. Multiple factors are attributed to those challenges, including the quality of recipient oocytes, the donor cell type, the genome editing method, the cloning procedure, and the adequate recipient conditions to maintain pregnancy. Therefore, effort must be undertaken to minimize inefficiencies at each step of the cloning procedure. In this sense, this review will

address the state of the art and the main challenges in creating an innovative pig model, covering procedures from ovary collection to embryo transfer.

2 Cloning techniques

Two different procedures have been used for the production of cloned pig embryos. The first one is the traditional method of cloning, also known as somatic cell nuclear transfer (SCNT), and the other one is the handmade cloning (HMC). Both have advantages and disadvantages, which will be further explained ahead.

2.1 Somatic cell nuclear transfer

The traditional method of cloning, the SCNT, is used in the majority of mammalian nuclear transfer laboratories and involves three major steps: enucleation, injection/fusion, and activation [30]. First, the oocyte nucleus is removed, then the donor cell nucleus is injected or fused with the enucleated oocyte. Lastly, the reconstructed embryos need to be activated and transferred into surrogates to develop into an individual that will be genetically identical to the one from which the nuclear material was derived [31]. The first report of cloned piglets made with this method was in 2000 [32], three years after the birth of Dolly the sheep, which was the first mammal that have been cloned from SCNT [33]. Since then, a series of cloned mammals, including cow [34,35], mouse [36,37], goat [38], cat [39], and non-human primate [40], have been produced.

SCNT provides an excellent opportunity for utilizing genetic engineered cells as donors for the generation of humanized disease models in large animals [30]. This technique utilizes primary cell culture for nucleus donation, and, during this *in vitro* phase, multiple genes can be inserted or deleted by genome editing technologies, such as CRISPR/Cas [27,41,42]. This process overcome problems with mosaicism, random mutations, and relative low editing efficiency associated with cytoplasmic injection [43,44]. In fact, SCNT combined with innovative genome

editing techniques have already been used to generate several pigs as biomedical models [12,24–26].

Unfortunately, the overall cloning efficiency remains low, as demonstrated by the fact that only 1-5% of the reconstructed embryos transferred to surrogate sows result in live offspring [45–47]. In fact, on average, in order to produce one edited live pig, it is required the reconstruction of 130 embryos [48]. Additionally, the requirement for expensive micromanipulators and skilled personnel to operate the equipment also hamper the success of the technique.

2.2 Handmade cloning

As mentioned above, the traditional method of nuclear transfer requires costly sophisticated tools, as well as highly skilled personnel. In order to avoid that, several laboratories adopted a relatively new and more economic approach, known as Handmade Cloning (HMC) [49]. The first successful HMC was achieved by Peura and colleagues [50], and subsequently, further modifications allowed the success of the technique [49], which has been used in many farm animals, including buffalo [51], sheep [52], horse [53], and pig [54].

HMC involves four major steps: zona pellucida removal, enucleation, fusion and activation [49]. First, *in vitro* maturation (IVM) matured oocytes are denuded and subjected to protease, pronase or hyaluronidase treatment for an efficient and harmless zona pellucida removal, which works even when large quantities of oocytes (up to 150) are digested together [55,56]. It is important to highlight that the fluidity maintenance of zona-free oocytes, as well as their suction into pipette should be done carefully, in order to avoid egg lyses [57].

Then, zona-free oocytes can be exposed to a cytoskeleton relaxant, demecolcine, for a membrane protrusion formation containing a mass of condensed chromosomes, which can be removed by bisection with an embryo-splitting blade under a stereomicroscope to obtain zona-

free hemi-cytoplasts [58]. As the reliability of this procedure is 96–98%, no further potentially harmful staining for the selection of chromatin-free cytoplasts is required [49]. However, if the polar body or nucleus are unable to be located, such as in pig oocyte with high lipid droplets formation, it is better to dissect the egg into two equal halves to generate cytoplasts, and screen for nuclear material, preferably by techniques such as harmless fluorescent observation of chromosomes in living oocytes [59,60].

Subsequently, reconstruction is achieved by exposing two hemi-cytoplasts to phytohemoagglutinin (PHA), which helps with attachment, and sandwiching the donor nucleus between them two, making sure that they have a wide contact area [57,61]. After attachment, reconstructed embryos are fused either by single step electrofusion or two step electrofusion [57]. Finally, chemical activation of reconstructed cloned embryos in ionomycin and N-6 dimethylaminopurine (6-DMAP) [62] is carried out, inducing calcium release and suppressing maturation promoting factor (MPF), consequently, releasing the reconstructed oocyte (enucleated oocyte + donor nucleus) from metaphase [57].

After activation, in order to efficiently culture individual embryos until blastocyst stage and avoid aggregation, an inverted sugar-loaf-shaped microwell “well-of-the-well” (WOW) system should be used, providing three dimensional blastomere arrangements [63]. Coupled with this system, media with elevated macromolecule content [49], or even an artificial zona pellucida can also be implemented [64]. Subsequently, those embryos are transferred into synchronized surrogates. In the porcine specie, however, there are some particularities that make achieving pregnancies and offspring more demanding. Some examples of that include the requirement of an increased number of quality embryos to establish early pregnancy [32,65,66], and the extended IVC requirement of HMC (until blastocyst stage), precluding early stage embryos transfer [54].

The technique has gained popularity due to its simpler and faster procedure [49]. Time and productivity are essential in cloning, and with HMC around 30-50 transferable-stage embryos can be produced from 200 slaughterhouse-derived oocytes every 3-4 hours [49]. In addition, other advantage of HMC is that zona-free blastocyst do not hatch, therefore, zona hardening which usually occurs during *in vitro* culture of SCNT embryos, is avoided [67]. Another advantage of handmade cloning is that the developmental rates with handmade cloning have been reported to be comparable or even higher than conventional cloning technique in pigs [53,68].

Some limitations of the technique include the loss of up to 50% of cytoplasmic volume during manual bisection of oocytes, which could potentially hamper reprogramming, and have adverse effects on the developmental competence of embryos [51]. With that in mind, the procedure has been updated, and now two enucleated demicytoplasts, instead of one demicytoplast, are utilized for fusion with the somatic cell and production of reconstructed embryos, which compensate the previous loss [69]. However, this approach introduces to other issues, such as mitochondrial heteroplasmy, in which conflicting publications concerning its effect on development of cloned embryo have been reported [70,71]. In addition, there is a requirement for numerous oocytes [49] in HMC, which can be overcome by utilizing slaughterhouse-derived ovaries.

One potential benefit for HMC development is automation, which can be achieved through use of microfluidic technology in a 'cloning biochip' device, as suggested by Vajta and colleagues [72]. Almost all the steps required for HMC can be performed in microchannels [72], what is different to SCNT, where automation seems to be impossible. For that, to develop a method that physically and spatially orientates mammalian oocytes in a specific position for enucleation on the biochip is fundamental [73]. One possibility could be the use of a specific monoclonal antibody anti double strand DNA (anti-dsDNA) conjugated to magnetic nanoparticles. Thus, posteriorly to binding, an electromagnetic field could be applied to

orientate the nucleus and polar body (which has most DNA of the oocyte) in a certain direction, facilitating therefore the automated enucleation.

However, some limitations regarding automation of HMC still hamper its practical use. Between them, we can cite the integration of the individual steps into a production line, and the occurrence of gas bubbles in the channels during incubation [57]. Hence, technical advances are still needed in order to enable the production of first-class embryos by highly standardized and reproducible procedures.

2.3 Nuclear reprogramming strategies

Several hypotheses have been proposed for the high loss of cloned piglets, mainly including placental and vital organs malformations usually derived from incomplete somatic cell nuclear reprogramming [74–77]. In the cloning procedure, a reverse differentiation known as dedifferentiation is necessary to redirect matured cells (donor cell) to a totipotent embryonic stage [78,79]. The clone's chromatin needs to pass on epigenetic changes, such as X chromosome inactivation, histone acetylation, DNA methylation, and remodeling of chromatin associated proteins, to make its structure similar to the embryonic chromatin [80–83].

Therefore, the improvement of the efficiency of SCNT has become a hot spot of current research, and the transcriptome and epigenetic analysis of SCNT embryos through low-input sequencing techniques have already revealed molecular defects, providing pathways to overcome them [84–86]. For instance, a link between SCNT and X Chromosome Inactivation (XCI) was established through a transcriptome comparison of single mouse SCNT blastocysts with sex-matched IVF counterparts, which discovered that many X-linked genes were repressed in SCNT embryos regardless of sex [87]. This disclosure led to an investigation of Xist, a long non-coding (lnc) RNA that is transcribed from the silenced X chromosome and responsible for dosage compensation of X-linked genes in XCI [80].

This lncRNA was found to be aberrant activated in pig SCNT embryos [80], and its DNA methylation level was reported to be lower than that in IVF embryos, irrespective of the sex, upregulating Xist expression and consequently repressing the transcription of numerous X-linked genes [80,88,89]. Moreover, this resulting abnormal XCI pattern seriously affects the development of cloned fetuses and placentas [90]. As a natural extension to the previous findings, targeting XCI became another strategy for improving developmental potential in SCNT, resulting in two approaches: the injection of short interference RNA (RNAi) of Xist into reconstructed embryos, and Xist knockout [87,91].

In porcine SCNT embryos, a previous report showed that RNAi-mediated Xist repression only slightly improved the survival rate of cloned pig embryos [91]. Subsequently, Ruan and collaborators described that the abnormal upregulation of Xist in pig was not restricted to early stage of pre-implantation, but also in the post-implantation stage, further suggesting that the RNAi treatment is not applicable for improving SCNT embryo development in pigs [80]. On the other hand, Xist knockout in donor cells normalized aberrant gene expression in cloned embryos, enhanced long term development capacity and increased 6.9 times the cloning efficiency when compared to wild-type cells [80]. However, it is important to notice that this strategy has limitations, since it is only applicable to male clones, limiting donor cell choice, furthermore, there is a possibility of an additional silencing mechanism independent of Xist exist in pigs, as it was suggested in mice [87,92].

Another discovery derived from transcriptome and epigenetic changes analysis was that the level and state of several histone acetylation marks in SCNT embryos chromatin are different from those from IVF embryos. In fact, deacetylation of histones was commonly found in the transferred cell nucleus [81]. Hence, several histone deacetylase inhibitors (HDACis) have been tested in order to improve cloning efficiency, including Trichostatin A (TSA), scriptaid, and Valproic acid (VPA) [74,81,93–97]. Treatment of cloned embryos with TSA improved both pre-

implantation development and offspring rate in pigs [81,93,95], however, the effect was limited, since treated cloned piglets showed higher percentage of abnormalities, perhaps due to the long-term exposure of embryos to TSA [95].

In addition, VPA treatment of pig SCNT embryos enhanced embryonic development, but impaired the survival to adulthood when compared to those from control embryos [98]. Scriptaid treatment, on the other hand, improved the success rate of pig cloning to full term, and improved the histone acetylation in a pattern similar to that of the *in vitro* fertilized (IVF) embryos [95]. Overall, although the mechanism of HDACi remains unknown and its beneficial controversial, the idea of a genome wide reprogramming should be pursued and improved.

Following the genome wide strategy, improving DNA demethylation in cloned embryos has been desired, since the genomic DNA of donor somatic cells is highly methylated, therefore leading to the continuous expression of tissue specific genes and inefficient activation of genes, essential for the embryonic development [82,99,100]. One way to overcome the aberrant DNA methylation is by recapitulating the pattern of normal fertilized embryos through DNA-demethylating agents or by DNA Methyltransferases (Dnmts) including Dnmt1 and Dnmt3l gene silencing [82,100–102]. Actually, Dnmt1 knockdown by RNA interference (RNAi) improved the methylation reprogramming of pluripotency and tissue specific genes in cloned pig embryos, suggesting that Dnmt1 in donor cells may impair development of the future reconstructed embryo [82,102].

Knockout strategies have been used in murine Dnmt1, however leading to embryonic lethality [103]. In contrast, Dnmt3l knockout in murine somatic cells increased gene-specific DNA methylation and histone modification reprogramming, as well as developmental competence, potentially due to a more accessible chromatin state and reduced HDAC1 activity [102]. It is

expected that this method will be applied to other mammals, including pig, in the near future, which could bring satisfactory results.

Another distinct detailed analysis of donor cells and reconstructed embryos, now with next generation sequencing, identified histone H3 lysine 9 trimethylation (H3K9me3) of the somatic cell nucleus as a major barrier to reprograming [85]. These disrupted histone modifications affect chromatin accessibility, leading to disordered expression of genes required for the normal development of cloned embryos, resulting in low cloning efficiency [104–106]. H3K9me3 removal through injection of histone lysine demethylase 4 (Kdm4) mRNA in pig cloned embryos overcome that problem, displaying a significantly higher blastocyst rate and total cell number than the control group [107]. However, Kdm4A injection significantly elevated Xist expression, which would hinder the developmental capacity of pig cloned embryos [80]. Therefore, a transcriptional repression of H3K9me3 gene by the dCas9-KRAB system [108], which offers reversible inhibition at the DNA level, may be the way of breaking that reprogramming barrier.

A further disclosure of epigenetics studies reported that the expression of protamine 1 (Prm1) alone is sufficient to compact sheep somatic donor nucleus in a reminiscent shape of those of spermatids, which is, in fact, the only nuclear formation that oocyte has evolved to deal with [83,109,110]. Furthermore, Prm1, when binding to the DNA, replaces somatic histones, including H3K9me3 [83]. This provide a promising approach for improving pig cloning efficiency, and research effort must be made to achieve that.

Overall, precisely understanding of the epigenetic changes that occurs during embryo development might be the key progress to improve pig cloning efficiency. Coupled with that, we might be able to achieve high-yield SCNT cloning outcomes by targeting fewer epigenetic errors than originally anticipated. For this purpose, additional research must be made in order

to test whether the reprogramming barriers and treatments identified in mice and other mammals are conserved in pigs.

3 Recipient cytoplasm and its epigenetic reprogramming capacity

The efficiency of SCNT is largely influenced by the reprogramming capacity of the recipient cytoplasm, as they play a critical role in the activation and subsequent development of the embryos [111,112]. In mammals, oocytes at metaphase II (MII) and one-cell zygotes have been used as recipients for nuclear transfer. Initially, experiments indicated that the zygotic cytoplasm could not support efficient reprogramming of the transferred nuclei [113], though posteriorly, with a slightly different protocol, researchers were able to achieve offspring formation with zygotic cytoplasm as recipient [114–116].

The updated protocol includes a breakdown of zygotic pronuclear structures before or upon enucleation, resulting in the release of pronuclear contents into the zygotic cytoplasm [111]. It contrasts with previously used protocols, in which enucleation was performed through the removal of intact pronuclei [113],overall, leading to the hypotheses that one or more factors that are necessary to support nuclear reprogramming are localized in the pronuclei [114]. However, even with these recent advances, the reprogramming process in zygotes remains much less efficient than in MII-oocytes [111]. Furthermore, the ooplasm of MII oocytes has already been reported to contain all the necessary factors to efficiently reprogram the differentiated somatic cell nuclei and support embryonic development [111]. Consequently, they became main choice when concerning recipient cytoplasm.

3.1 Ovary collection

Oocytes can be retrieved *in vivo*, from hormonally stimulated females, or post-mortem, from slaughterhouse ovaries derived from nonstimulated females [117,118]. Generally, ovaries collected from local slaughterhouses are the main source of oocytes for *in vitro* embryo

production research, including cloning techniques, even though it suffers from absence of information about health history, age and breed. Thus, ovaries must be transported from the slaughterhouses to laboratories, and even so, the ideal situation would be to proceed to IVM immediately after the collection, but in many situations, slaughterhouses are several kilometers away from the laboratory, and ovaries must be preserved in thermal recipients for a long period of time before follicular aspiration.

Actually, this long transport time may adversely affect oocyte quality in terms of nuclear maturation and developmental competence [119]. Instability of temperature seems to be a key factor on this outcome [120]. Moreover, immature oocytes are particularly sensitive to their environment, hence, its viability and quality - which is essential factor for cloning procedures - are directly proportional to appropriate storage conditions during ovary transport [121].

Moreover, in excised ovaries, lack of blood circulation will eventually lead oocytes to undergo ischemic condition [119]. Numerous studies have shown that it generates production of deteriorating conditions for the oocytes, such as low oxygen tension and accumulation of toxic metabolites [119,122]. Consequently, the hostile environment will end up triggering programmed cell death [123].

In fact, in the porcine species, short time and temperature maintenance seem to be the most adequate transport conditions of ovaries in order to keep the oocyte quality. More precisely, the ideal storage ranges from 25-35ºC for 2-3 hours [124,125]. In contrast, the preservation of ovaries at 15ºC, or for periods longer than 6 hours, decreases oocyte maturation and embryo development rates [125,126].

In order to alleviate these adverse effects, and be sure that the temperature will be stable throughout the whole process of ovary collection and transport, our group developed a thermal container used for transport and storage of ovaries [127]. The container has a bipartition with two

independent holes, one for the water at desired temperature, and other for the storage of the ovaries. In addition, it consists of two caps designed to seal the compartments and contains two supports for removable handles, in order to facilitate in the collection process and the handling of the container.

3.2 Oocyte IVM

Pig oocytes are arrested at the diplotene stage of the first meiotic division on the ovarian follicles [128]. Upon appropriate stimulation, they undergo resumption of meiosis, characterized by germinal vesicle breakdown, chromosome condensation, formation of the first meiotic spindle, expulsion of the first polar body and arrest in metaphase of the second meiotic division, which ultimately define oocyte maturation [128]. IVM in porcine oocytes demand incubation in supplemented media for 44 h at 39°C in 5% CO_2 [129].

Subsequently to IVM procedure, matured oocytes are usually selected by morphology, when the number of cumulus cell layer surrounding the oocytes, their compactness, and ooplasm homogeneity are observed. The selected oocytes are then submitted to the enucleation protocol, for a complete removal of nuclear genetic material [112]. As mentioned above, diverse methods can be used for it, but most importantly, the competence of the oocyte has to be maintained to possible extent.

4 Donor cell

Posteriorly to choosing the right recipient cytoplasm, another important aspect to be analyzed is the source of the donor nucleus. Selection of donor nucleus is an extremely important aspect when aiming to increase nuclear reprogramming and cloning efficiency [130,131]. Numerous publications observing and comparing the effect of the type of cell, cell cycle stage, and the quality on embryo production have been published [132,133].

Regarding the porcine species, fibroblasts (fetal and adult) are traditionally used as donor cell choice, mostly due to easy sampling, isolation, and *in vitro* maintenance, offering various advantages [112,134]. The most important to generate an innovative animal model is the opportunity for genetic modification and cell population selection before embryo reconstruction. More precisely, fetal fibroblasts might be one of the best choices for efficient SCNT in pigs, since they present higher efficiency of piglets born, as well as lower rate of developmental abnormalities when compared to adult fibroblast [135]. However, prenatal cloning limits the knowledge of the animal's future phenotype, which could possess some abnormality, ultimately influencing the success of the cloning technique. Thus, electing an adult fibroblast with the sought phenotype and no abnormalities related could be a better approach.

4.2 Cell culture

Establishing cell banks seems are highly important to maintain the specific genotype to produce standard cloned embryos and future animal models, however, recent research suggests that the passage number of the donor cells affects the success of SCNT [136]. In fact, first passage cells would be the ideal donors, on the other hand, properly done GE and screening demand time and consequently cell passages, therewithal preferably donor cells with four to six passages should be utilized [137]. Jin et al. reported that cells with seven and eight passages did not produce piglets, hampering the idea of cell bank establishment [137]. In addition, cell confluence is another key process in donor cell choice: high confluence cells (>90%) results in higher pregnancy and delivery rate and overall cloning efficiency when compared with lower confluences (60%-89%) [137]. Ultimately, the cloning technique is deeply affected by donor cell choice, and developing an optimized technique will certainly bring benefits in terms of animal model generation.

5 Simultaneous coordination

Subsequently, a further major factor needed to increase nuclear reprogramming capacity and, thereby, cloning efficiency is the simultaneous coordination between the cell cycle of donor nucleus and the recipient cytoplasm [138,139]. In fact, this is utmost important to maintain correct ploidy of the reconstructed embryos and for enabling proper development [139]. Thus, as mentioned before, the most appropriate choice of recipient is the ooplasm of MII oocytes and the donor nucleus are frequently somatic cell (e.g. adult fibroblast), so the synchronization protocol will follow that.

When an oocyte becomes arrested at metaphase II (MII), maturation promoting factor (MPF) activity is high, and along with that, any nuclei that are transferred into it undergo nuclear envelope breakdown and premature chromosome condensation (PCC) [139,140]. Thereby, previous reports had shown that the most suitable donor nuclei for this environment must be quiescent donor cells in the G0 or arrested in the G1 phases of the cell cycle, since such cell cycle phases are considered as more prone for proper reprogramming [139,141]. Moreover, if the recipient ooplasm is activated prior to transference, MPF activity decline, and PCC is avoided, leading to formation of an "Universal Recipient", in which all nuclei, regardless of their cell cycle stage, undergo co-ordinated DNA replication [142].

Furthermore, different procedures for the cell cycle synchronization in G0/G1 phase of donor cells are available. Serum starvation, contact inhibition by cell confluence, and use of chemicals like cycloheximide, DMSO, roscovitine and nocodazole are the most commonly used. In porcine cloning, contact inhibition under total confluence conditions seems to be the most frequently used method [143–146]. In fact, this approach is the least stressful since it consists in culturing cells for approximately four days, or until a high confluence (90%) is reached, being extremely efficient, even after freezing and thawing procedures [137].

6 Embryo culture and transfer

Reconstruction, fusion and activation of the new presumable zygote must be performed according to the chosen cloning technique (SCNT or HMC), as previously described. Thus, subsequently to activation, the donor cell genome will enter to G1 phase and will form the nuclear membrane, then, through nuclear expansion, the cloned embryo will form pseudo-pronucleus (PPN), much larger than the original donor somatic cells, and in a varying number (usually 1 or 2) [147]. Depending on the transfer protocol, the cloned embryos will be cultured until different stages of development.

Two main transfer protocols are used for pig cloned embryos. The first one is based on transferring reconstructed embryos cultured for 1 to 2 days into the oviduct of recipients, and the second one consists of transferring blastocysts (5 days after de cloning procedure) into the uterine cavity of recipients [131,137]. Both approaches have reported satisfactory results, however it seems that the extensive IVC enable the identification of embryos in which the embryonic genome is activated, since genomic activation usually occurs at the four-cell stage in pigs [137,148]. Also, it mimics more closely what occur in natural conception, allowing better embryo-endometrium synchrony, and consequently higher implantation chances [149].

Recipient estrus synchronization is another important factor, since it plays a critical role in maintaining pregnancy [150]. The protocol will be initiated according to the scheduled date for transfer, and although the estrus can occur naturally, chemical induced synchronization was reported to achieve higher pregnancy rates when compared with untreated recipients [137], probably due to its better precision. Usually, prostaglandin and combinations of prostaglandin and gonadotropins are used for induction [112].

Moreover, due to the poor developmental capacity that cloned porcine embryos still exhibit, a sufficiently high number of embryos has to be transferred to each recipient in order to compensate that, and thus, manage to establish and maintain pregnancy. Usually, more than

100 cloned embryos are transferred per recipient, which allows *in vivo* selection of the developmentally competent embryos [32,65,66]. Along with that, the restriction in uterine space and the competition among embryos for nutrients and biochemical factors also limits the number of fetuses developing to term, which usually end up remaining slightly more than four [151,152].

In addition, the breed of the cloned embryos and their recipients have been suggested to interfere with cloning efficiency, especially when miniature pigs are involved, since the uterus size and birth weight are extremely lower than domestic pigs. Initially it was proposed that pregnancy and delivery rates were significantly increased if the transferred cloned embryos and their recipient were from the same breed [47,153]. In contrast, another study reported that when diverging breeds were used, highest pregnancy rate, delivery rate and largest litter size were obtained [137]. Overall, additional studies are required to analyze the best combination of embryo/recipient breed, however, most importantly, the breeding environment for miniature pigs and domestic pigs diverge, and that must also be taken into account for the selection of the recipient pig.

7 Recloning

Finally, after successfully generating the desired animal model, an alternative technique known as recloning, or serial nuclear cloning, can be utilized for maintaining the animal lineage, ensuring reproducibility and stable transgene expression [154,155]. Recloning is based on utilizing a cloned embryo, fetus or animal cell as donor for a new round of cloning [156]. Moreover, encouraging results regarding recloned pigs production have been published [157–159].

In fact, recloned transgenic pigs maintain normal reproductive performance and stable genetic transmission capacities, as well as have been reported to be produced at comparable efficiencies to standard cloning [157,159]. In addition, Ahn and collaborators demonstrated that the loss of an GE pig could be rescued by recloning, successfully generating a healthy offspring

[158]. Overall, the technique is a promising tool for GE animal model maintenance, however, still presents drawbacks, which are probably linked to the various previously discussed factors, limiting the cloning procedure.

8 Perspectives

Major improvements have already been done in every step of the pig cloning procedure, however, despite that, its overall efficiency remains low. With that in mind, and in order to further refine the technology, advances must be done to accomplish commercial production of this animal models. In fact, a considerable potential market would be personalized medicine, in which an animal would be generated containing the patient's mutation in its genome, for development of a specific therapy screening with individual results. For this purpose, full annotation of the porcine genome sequence has to be completed.

Additionally, analyzing the global gene expression patterns of cloned pig embryos trough RNASeq technology, and comparing them with genotype- and sex-matched controls produced by *in vitro* fertilization under the same environment will further improve our ability to identify important targets. Moreover, lncRNAs should also be focus of research, since exploring and clarifying their underlying molecular mechanism on epigenetic reprogramming will probably lead to improvements in cloning efficiency. Along with that, molecular markers of somatic donor nuclei should be identified, characterized and posteriorly utilized as criteria for selection.

Furthermore, the improvement of techniques that directly introduces the desired mutations into pig zygotes and, as a result, does not require the epigenetic reprogramming of a donor somatic cell nucleus will probably also enhance cloning yields, as it skips the most controversial step in the procedure. An example is GEEP (gene editing by electroporation of Cas9 protein), which is based on electroporation of Cas9 protein into zygotes resulted from

normal IVF, achieving high mutation rates (up to 90%) and as it does not require advanced skills and expensive micromanipulator, and demand considerably less time, the technique has large-scale potential [160]. Furthermore, the improved genome editing via oviductal nucleic acid delivery (i-GONAD) is another example of promising technique, since it consists in injecting the Cas9/gRNA complex directly into the oviduct of pregnant animal, followed by in situ electroporation, which is also advantageous due to avoiding ex-vivo embryo handling [161]. However, i-GONAD have not been tested in pigs yet, only mice, consequently research must be done for obtaining results in this species.

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4.2 Artigo 2 - Recloning animals: an underestimated technique

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Recloning animals: an underestimated technique

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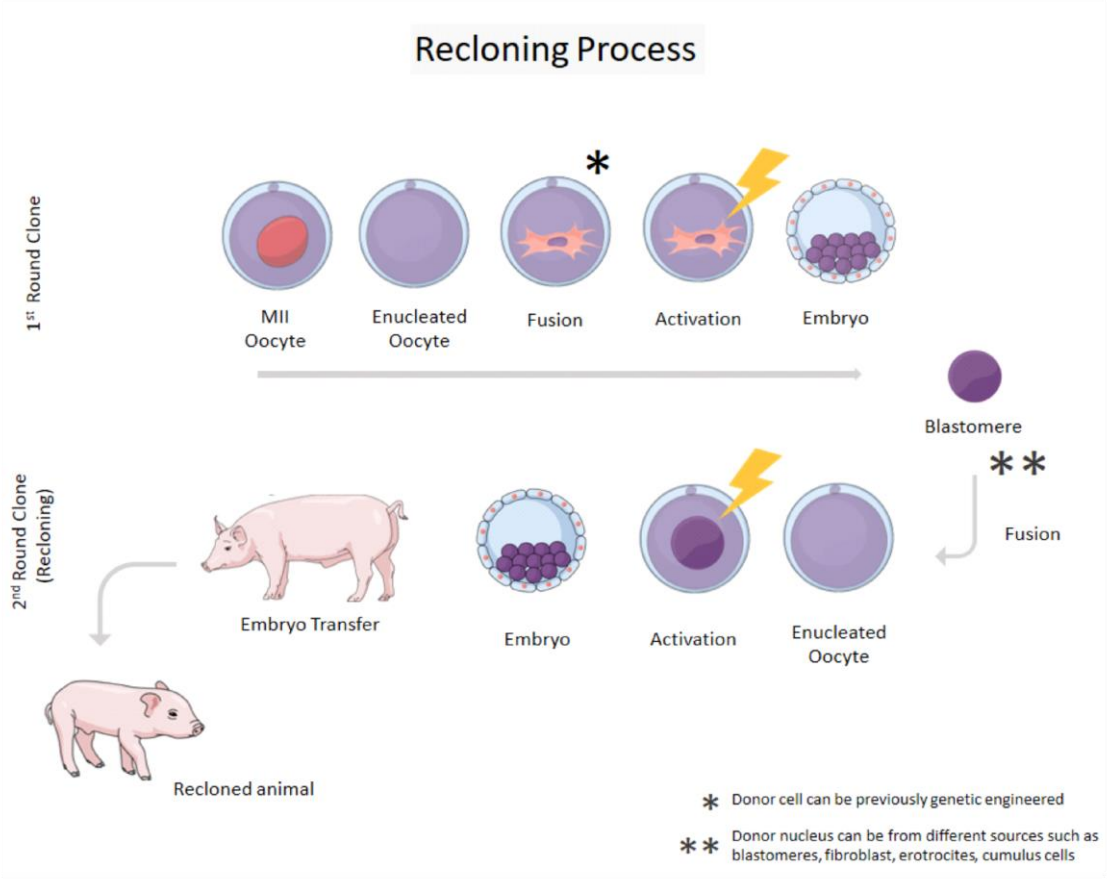
Recloning animals: an underestimated technique

Despite intensive efforts, the efficiency of SCNT in producing viable offspring has been low. Abnormalities associated with genomic reprogramming errors and the short lifespan of donor cells limits multiple genetic modifications. Based on that, the recloning method has been suggested, in which a viable reconstructed embryo or a cloned animal could be used as nuclear donor for a second round of SCNT. Different protocols involving pre-activation of cytoplasm or the establishment of the cell cycle stage of the donor cell, demonstrated levels of efficiency in the technique. In this review, we addressed the advances in the recloning process and highlighted the mechanisms involved in reprogramming resistance and consequently low cloning efficiency. Also, it is mentioned the importance of the use of genetically engineered animals as biomedical models to perform accurate studies following the 3R's principles, and the role of recloning for maintaining these generated animals.

Keywords: Animal model maintenance; HMC; SCNT; Serial nuclear transfer.

Graphical abstract:



Introduction

The large mammals cloning process was first achieved in 1986, when sheep embryos were successfully produced by nuclear transfer using embryonic cells as nuclear donors [1]. However, only one decade later mammal cloning was highlighted, after the birth of Dolly the sheep, generated by SCNT (somatic cell nuclear transfer), which is an emerging technology that consists of three basic steps: the oocyte enucleation, the injection of the donor cells (or nuclei) and the activation of the reconstructed oocyte [2]. The cloned embryos, in most protocols, are temporarily *in vitro* cultured and then transferred into a synchronized recipient [3].

Despite intensive efforts the efficiency of SCNT in producing viable offspring has been low. Abnormalities associated with genomic reprogramming errors and the short lifespan of donor cells limits multiple genetic modifications [4]. Based on that, Willadsen and collaborators [5] suggested the recloning method in which viable reconstructed embryos could be used as nuclear donors in a second round of SCNT. Recloning or serial nuclear cloning involves the transfer of a nuclei, from a blastomere or a somatic cell derived from a previously nuclear transferred embryo/animal, to an enucleated fertilized zygote or to an ooplast, which will be, then, parthenogenetically activated [5]. Different protocols involving pre-activation of cytoplasm or the establishment of the cell cycle stage of the donor cell, demonstrated levels of efficiency in the SCNT approach [6,7].

Recloned embryos have several potential applications: for basic studies (to understand the effects of progressively accumulating somatic mutations on development, health, and reproductive performance[8]), for resurrection and maintenance of disease models (recloning transgenic animals alive or post mortem[9]), for agriculture (increasing productivity and improving animal welfare[10]), xenotransplantation (multiple genetic modifications and reproducibility[11]), and regeneration of cell lines (when cells approach senescence[12] or when

the cell lines are in limited supply[13]).

Cloned and recloned animals have been reported to be produced at comparable efficiencies [14,15]. Cao and collaborators demonstrated that reproductive characteristics of recloned transgenic pigs were not significantly different from those conventionally bred[16]. Also, Ahn and colleagues[11] showed that fibroblasts derived from a piglet that died due to respiratory distress syndrome and cardiac dysfunction could be successfully used to produce a healthy recloned piglet. Although they proved that serial cloning is possible, other authors have reported fetal and postnatal losses as well as placental abnormalities [15,17], and a decrease in cloning efficiency over generations [8,18–21].

In this review, we address the advances in the recloning process and highlight the mechanisms involved in reprogramming resistance and consequently low cloning efficiency [22]. In addition, we highlighted the importance of the use of genetically engineered animals as biomedical models to perform accurate studies following the 3R's principles, and the role of recloning for maintaining these generated animals.

Epigenetics

Epigenetics was described as "the branch of biology which studies the causal interactions between genes and their products, which brings the phenotype into being" [23]. It consists of a link between phenotypic and genotypic events which alter patterns of gene regulation without altering the DNA sequence [24]. Understanding the epigenetics mechanism for normal development and maintenance of gene expression on tissue specific locus is essential [25]. Epigenetic modulators help to reprogram cells and restart the totipotent status [26].

In the cloning procedure, a reverse differentiation known as dedifferentiation is necessary [27]. The clone's chromatin needs to pass on epigenetic changes to make its structure

similar to the embryonic chromatin [22]. Dedifferentiation process includes changes in DNA methylation, histones, remodelling chromatin associated proteins, transcription factors, imprinting, X chromosome inactivation, genome stability and silencing of retrotransposons[27,28]. These changes happen differentially depending upon the cell type, stage and time, with each cell type having a specific profile, and consequently, a specific expression [22,28]. Overall, it is an extremely complex process that involves multiple steps [29].

Among the changes that occur in dedifferentiation, the best characterized epigenetic modification is DNA methylation in CpG islands [30]. It is responsible for transcriptional silencing, imprinting process, heterochromatin formation, x-inactivation and genomic stability [31]. This process is dependent on the DNA methyltransferases activity (Dnmt) which maintains the pattern of methylation following DNA replication. Another mechanism that contributes to epigenetic modulation is the posttranslational modification of histones by acetylation, ubiquitination, methylation and phosphorylation. This process leads to changes in chromatin structure and in protein-protein interactions regulating gene transcription [32].

Also, an epigenetically regulated compensation process known as X chromosome inactivation, results in a random silencing of one X chromosome in female mammals [33]. During embryo development, on the cleavage stage, both the X chromosomes are active. When the transcription starts, a histone methylation process occurs resulting in gene silencing [34]. This mechanism is potentially affected in clones, since a high rate of aberration in the X chromosome has been observed [33].

Similarly, non-coding RNA (ncRNA) is an important regulator of epigenetic phenomena, and acts either in heterochromatin or in euchromatin [35]. There are several types of ncRNA, among them microRNA, which acts in cell proliferations and differentiation and shows promise in biomedical applications; the small interfering RNA, which has function on genetic

silencing; and small nuclear RNA which acts in post-transcriptional modifications, mainly on ribosome synthesis [36–38]. Most of these have a role in genetic silencing and their action pattern can pass by across the cell division [39]. Overall, there are several epigenetic processes acting in the cloning and recloning process that must be taken into account.

Reprogramming occurs slowly and progressively, so cloned embryos show a high incidence of developmental abnormalities when compared to fertilized embryos [29]. Cloning process have resulted in a high rate of spontaneous abortion, peri or postnatal death in different species, and in a low development of healthy young animals [40]. External factors such as *in vitro* manipulation and culture media composition, inherent in the process, can alter the epigenetic status [27]. Also, the origin of the donor cell and their differentiation status have been strongly associated with clone development progression until blastocyst stage [41]. Based on that, it has been thought that successive rounds of cloning can extend chromatin exposure to epigenetic factors leading to an enhancement in reprogramming process [42,43].

Rodriguez-Osorio and collaborators did not observe significant difference when compared the global transcriptome of bovine blastocysts produced after one round of cloning versus blastocysts produced after four consecutive rounds of cloning [44]. However, a set of genes were misregulated in recloned embryos when compared to IVF embryos. Genes involved in cytoskeleton rearrangement and cell shape demonstrated to be upregulated in recloned when compared to IVF embryos. While chromatin remodelling and stress coping-related genes showed to be upregulated in IVF embryos [44].

In swine, researchers observed that after SCNT a piglet presented an abnormal appearance, probably due to a failure in the epigenetic regulation during the SCNT process. To prove this cause, recloned piglets until the third generation were made to confirm if this phenome was due to errors on epigenetic or damage on DNA. As a result, no phenotypic

alteration were observed, proving that it probably occurred due to epigenetic dysregulation only in the first round of cloning [15].

In another study, Ahn and collaborators[11] reported the production of a transgenic piglet, after they used postmortem ear skin fibroblasts from an αGgene-targeted cloned piglet as nuclear donor cells (died due respiratory distress syndrome and cardiac problem) for the second round of SCNT. The recloned piglet produced confirmed the presence and expression of the αGgene-targeted gene by PCR and Western blot analysis. Also, no health problems were observed showing that the issue did not pass to prole. This methodology can prevent the loss of great genetic value animals [11].

Donor cell

Discussions arising the effects of the differentiation state of a transplanted donor karyoplast and its ability for reprogramming have supported the theory that blastomeres have lower methylation levels and are in the undifferentiated state, thus becoming a good alternative [12]. However, Uhm and collaborators [45] demonstrated that recloned embryos originated from blastomeric donor cells did not show any improve on *in vitro* development and presented a slower nuclei remodelling process, when compared to somatic cell nuclei. Furthermore, the limited number of cells in preimplantation embryos can be an obstacle to the generation of identical progenies from a single donor embryo [10,46]. To overcome this problem and expand the potential of this technique, the use of embryonic stem cells as a source of donor nuclei have been proposed, however its application was still limited [47].

Regarding cell type influence, Galli and collaborators suggested the use of leukocytes as nuclear donors in normal SCNT, as they can be easily collected and cryopreserved from all mammals (regarding specie, age, or sex) [48]. Additionally, leukocytes DNA seems to be more protected from environmental radiation and demonstrated less karyotype abnormalities due the

fact that they are not subjected to *in vitro* culture, which is an advantage since Zakhartcheko[43] published that extended culture induces a marked reduction in the efficiency of bovine NT (nuclear transfer).

Contradictorily, according to Wolf [10], the ability to perform serial nuclear transfer from a cell population that can be maintained in culture offers numerous advantages such as the opportunity for genetic modification and cell population selection before embryo reconstruction. However, this approach is uncertain, since antibiotic selection of genetic modified somatic cells are not always accurate, which can result in population contamination with wildtype cells, ultimately leading to undesired birth of a wildtype animal [49–51].

As an alternative, adult and fetal fibroblast cells have been used as nuclei donors. Adult fibroblasts have a finite life-span in culture, limiting its use for transgeneses purposes [12]. On the other hand, fetal fibroblasts, confirmed by Liu [52], have been demonstrated to be great candidates during SCNT, due to its high developmental competence [53,54], longer term survival and genetic stability in culture [55,56]. However, prenatal cloning does not allow the use of an existing adult animal, thus limiting the knowledge of its future phenotype.

Therewithal, after electing the donor cell, other procedures need to be chosen, once researches focused in SCNT have shown that the cell cycle stage of the donor cell and the preactivation or not of the recipient cells affects the extent of development of the fused embryo. According to Campbell and collaborators, coordination between donor nucleus and recipient cytoplasm requires that either the nuclei is synchronized in the G1 phase of the cell cycle, which precludes their ability to re-replicate DNA, or that the introduced nucleus is prevented from undergoing PCC by preactivation of the recipient cytoplasm, reducing the level of MPF [7].

Nocodazole is an antineoplastic agent which exerts its effect in cells by interfering with the polymerization of microtubules [57], and has been described to increase morula/blastocyst

formation by the synchronization of blastomere nuclei in the G1 phase before fusion [58]. However, its use in serial cloning technique decreased the yield, which indicates a deleterious effect of the second treatment with the compound [58]. An alternative is cell cycle synchronization by contact inhibition under total confluence conditions, in which cells are cultured until a high confluence (90%) is reached [59]. Overall, protocols for producing embryos from nuclei arrested at any stage of the cycle have yet to be researched and established, as they would greatly facilitate the procedure.

Regarding the source of the ooplast, Wakayama [60] published that genetic heterogeneity between the donor nucleus and the recipient oocyte cytoplasm does not influence the quality of reprogramming and full-term development of cloned mouse embryos. Stice and collaborators reported that in cloning and recloning procedures, minimal cytoplasm removal during the enucleation process may enhance the final number of cells in clones, and consequently the effectiveness of the technique [18]. Thus, the effect of cytoplasm volume attracts researchers' interest in order to understand its implications in future embryo development [61].

The undesired failure in the process of recloning can occur by different factors and deepening the knowledge about the modifications that occur during this process can help in the development of a more effective technique. Development of a suitable media of culture, containing transcription factors and molecules that help the cloned embryo to complete its development, as well as adjusting the time of each step of the protocol and choosing the most appropriate cell type, are essential steps that should be improved and taken into account.

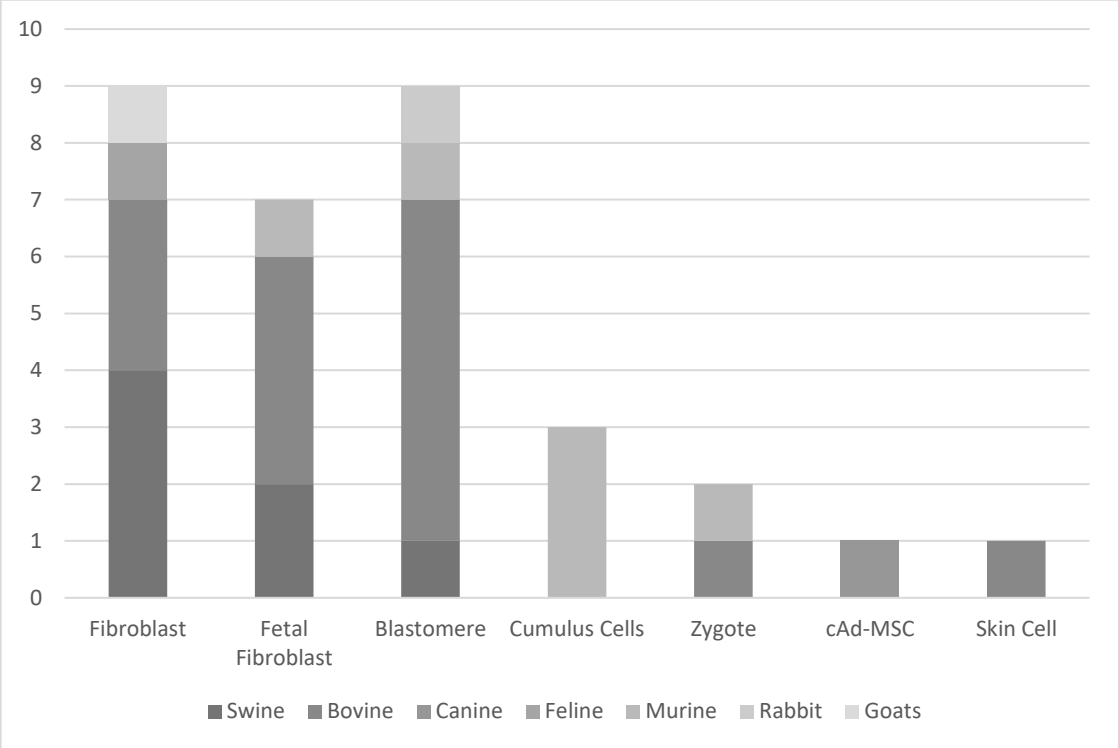


Figure 1. Different types of cells used for recloning in different animal species. CAd-MSC (adipose tissue-derived mesenchymal stem cells)

Recloning outcomes

It has already been reported that serial nuclear transfer could be performed without compromising production efficiency [42,43,60,67] or even improved the developmental competence of the SCNT embryos [66,68,69,76]. However, contradicting publications affirmed that the efficiency decreased proportionally as the number of rounds of nuclear transfer [8,18–21,62]. It could be argued, though, that the reason for opposite result reports is influenced by variation of donor cell types between recloning generations and between research groups, or even by the elected technique for the cloning procedure.

Failure in recloning could be associated with inappropriate synchrony between the cell cycle phase of recipient cytoplasm and donor nucleus [3], cumulative suboptimal embryo culture conditions [18], reduced viability of donor embryos [3], accumulation of genetic or epigenetic abnormalities over successive generations [60], and the inherent success rate of cloning being too low for it to be reliable over repeated generations [60]. Another factor related to the developmental

outcome of serial nuclear transfer could be the timing of maternal-zygotic transition (MZT), since it has been documented that its timing varies between different species [77,78]. And the failure of the recloning procedure could be either from any or all of these criteria, singly or in combination.

Ono and collaborators observed placental hypertrophy and defective differentiation of placental tissues in all of the cloned embryos, suggesting this as one of the factors related to the high neonatal death in serial cloned pups derived from somatic cells [17]. Similar abnormalities were observed in somatic cloned embryos, regardless of the species and cell types [12–14,17,47,79]. However, it is important to reiterate that this and other abnormalities such as increased birth weight, fetal overgrowth and prolonged gestation can also be detected not only after standard or serial nuclear transfer, but in other biotechniques such as PIVE or vitrification processes [80].

Contradictorily, many authors reported that the recloned offspring were healthy and fertile [8,20,48]. It has been even described the reiterative cloning of mice to six generations, in which successive generations showed no sign of premature ageing and no evidence of shortening of telomeres, in fact, they observed a slight increase of their length [21]. However, the success rate dropped, so that only one cloned mouse was produced in the sixth generation from more than 700 nuclear transfer attempts [21]. Wakayama and collaborators[60] also succeeded in carrying out repeated recloning in mice, obtaining more than 500 viable offspring from a single original donor mouse. Surprisingly, in this publication the cloning efficiency did not decrease over 25 generations, and no accumulation of reprogramming errors or clone-specific abnormalities were observed, leading to a suggestion that it could be possible to reclone animals indefinitely.

Kuroiwa and collaborators claimed that recloning is a feasible method for the production of large numbers of transgenic animals and the establishment of cryopreserved transgenic cell banks, since their study resulted in the survival of four healthy and phenotypically normal

calves [65]. Other groups corroborated to that hypotheses, publishing that the efficiency of nuclear transfer could be further increased by recloning, due to the more extensively exposure of the donor nucleus to conditioning factors in oocyte cytoplasm that is enabled by the additional round of nuclear transfer [69].

Recloned pigs

Swine have been widely utilized as a suitable biomedical model specifically for disease and xenotransplantation related studies due to similarities in organ size, anatomy, genetics, physiology and metabolism to humans [52]. Swine applications as biological models are not hampered by severe ethical objections as with primates, dogs and cats. Additionally, the short gestation period, large litter size, and early sexual maturity make swine relevant to agriculture production [81–83].

Hyperacute rejection (HAR) and acute vascular rejection (AVR) are the most common immunologic barriers for xenotransplantation [84]. Transplantation cross-species involves overcoming immunological and physiological barriers, due to molecular incompatibility between donor and recipient that leads to xenograft rejection. One example of this process is the hyperacute rejection (HAR) against the pig α 1,3-Gal epitope, synthesized by the α 1,3-galactosyltransferase (α1,3-GT). This response is mediated by natural antibodies and followed by complement system activation [50,85].

Research groups have been working not only on the creation of pig model expressing regulatory proteins or different glycosyltranferases which may be able to downregulate α 1,3-galactosyltransferase (α1,3-GT), but also in the development of a α1,3-GT knockout model [50]. It has been shown that when disrupting α1,3-GT [11], or inducing a heterozygous knock out or double knockout [85,86] phenotypic difference between the null α1,3-GT gene and wildtype were not shown. In addition, other researchers found the same results when serial cloning was

performed [87].

Recently, recloned pigs overexpressing the soluble human tumor necrosis factor (shTNFRI-Fc) and human hemeoxygenase1(hHO-1) were created in an attempt to protect against oxidative stress and inflammatory injury. The possible reduction in the resistance against xenograft rejection made these models suitable for xenotransplantations purposes. However, it was observed that piglets overexpressing these two genes demonstrated postnatal premature death and liver damage [88]. Overall, multiple genetic modifications are necessary for successful xenotransplantation from pigs, and recloning will likely become a popular method for the screening of these multiple-gene combination modifications animals [63].

In previous research performed by Uhm and collaborators, fetal fibroblast cells were transfected by using a LNb retroviral vector harboring enhanced green fluorescent protein (EGFP) reporter genes and subsequently injected into enucleated metaphase II oocytes. Reconstructed embryos generated by using a blastomeric nucleus from previously cloned embryos (produced by SCNT of transfected fibroblast nuclei) when compared to cloned embryos that had received transfected fibroblast nuclei did not show an improvement in transgenesis efficiency. Mosaicism was not observed in either group, and the pattern of nuclear reprogramming, as well as blastocyst and cleavage rates in recloned versus cloned embryos were similar. However, blastomeric nuclei seemed to undergo a slower reprogramming process than somatic cell nuclei [45].

Park and collaborators demonstrated that recloned embryos derived from EGFP expressing ear skin fibroblasts had a mosaic EGFP expression pattern. However, it was shown that transgenic pig production could be duplicated with no impairment in the transgene expression. Embryonic development to term and survival rates after the nuclear transfer process were still very low showing the need for studies in epigenetics factors associated with embryo

developmental ability [89,90].

Success in the recloning methodology using a genetic modified nuclei donor has demonstrated normal reproductive results regarding performance, integration of the transgene and genetic transmission in both sexes of recloned pigs [16]. It was already demonstrated that clones could be successfully produced after serial nuclear transfers without compromising telomere length and production efficiency [42]. The effects of recloning by using handmade cloning was evaluated between porcine cloned and normal adult fibroblasts, as well as cloned and normal fetal fibroblasts. When cloned adult fibroblasts were used as nuclei donor less abnormalities were observed and cloning efficiency was higher when compared to normal adult fibroblasts. However, when cloned fetal fibroblasts were used as nuclei donors the derived embryos showed higher rates of abnormalities and lower cloning efficiency when compared to normal fetal fibroblasts. The mechanisms underlying these effects is still unclear [52].

Zhao and collaborators used transgenic porcine fetal fibroblast cells to generate GTKO/hCD55/ hCD59 triple-gene modified pigs [63]. They were able to produce 12 clones, of which 2 fetuses expressed hCD55 and hCD59, and the one with the highest expression was chosen for recloning. As a result, they obtained 12 clones produced expressing GTKO and carrying hCD55 and hCD59, and observed a difference in the levels of expression between the animals, even though they were generated with great efficiency [63]. Another study carried out for the generation of recloned pig embryos was accomplished by Cho [62], in fact the embryos were carrying the beta-casein promoter/human granulocyte macrophage colony stimulating factor (hGM-CSF), and as expected, beta-casein expression was found only in the mammary tissue [91]. The first generation of transgenic cloned pigs showed abnormalities, probably associated with epigenetic reprogramming cell failure during development, however, they were not observed in the following recloned generations [62].

3Rs principle

For the advancement of science, the use of animals in research is essential as it enables the progress of new technologies. Allowing the understanding and development of treatments of diverse diseases, as well as the development and evaluation of products and prediction of their safety and efficacy [92]. In 1959, William Russel and Rex Burch developed the principle of 3R's work entitled "Principles of Humane Experimental Technique" in order to conduct animal research in a rational and ethical way [93].

The central idea of these principles was to reduce the number of animals used in research through practices that provide valid and consistent results. The principle of reduction is to reduce the number of animals used, guaranteeing valid results [94]. The refinement strategies refer to use procedures that bring less pain and distress to animals [95]. The third principle, the Replacement, attempts to use non-animal, tissues and cell culture or phylogenetically inferior species of live animals to replace animal use completely or partially [96].

In the beginning the efforts were to develop some alternatives instead of using animals in research [94]. In this sense, in vitro tests were largely implemented, but it could not answer in the same way that a whole body, once this approach uses a determined cell type and tries to mimic a group of organs which form the animal system [97]. Thus, in vivo studies continued to be quite performed, however less than 30% of traditional animal tests can predict the human response [98]. In this regard, the development of suitable animal biological models for the success of validation of new findings should be sought, since many promising in vitro assays fail in in vivo assays, which suggests that it is necessary to adapt, employing more robust biomodels that more specifically meet the needs of the sciences [99].

With the advance of modern genetics, genome editing techniques allowed the production of genetically engineered animals, and eliminated several barriers for the production

of more dashing and suitable biomodel [100]. In this sense, genetic engineering favors progress in biomedical research, once this leverages several areas, providing knowledge about functional genomics, permitting obtainment of pharmacological products, improving animal production, and allowing xenotransplantation with low risk of rejection [81]. Taking into account the practice of 3Rs for the use of animals and new alternatives in research, applying modern and contemporary techniques, contributes to animal science and welfare [94].

Conclusion and perspectives

The recloning technique represents a promising tool for development of important cell lineages and animals. Once it allows the maintenance of lineages of interest, especially those derived from specific mutations generated by genetic engineering, which attract great commercial and scientific interest. However, the technique presents divergent results when comparing species, protocols and elected donor cells. Such oscillations probably occur due to the various previously discussed factors, and may manifest from any or all of these criteria, singly or in combination.

From our knowledge, until now, there were no published articles using more up-to-date genome editing techniques, such as the CRISPR/Cas, in which it is possible to edit genes with just one step. Thus, the integration of recloning and CRISPR/Cas techniques would favour large-scale production of animals with a specific desired characteristic. Thereby, biomodels for the study of basic science on aging and reprogramming, as well as for applied science, directed to the testing of drugs, vaccines and diagnostic tests, could be developed. In addition, this innovative approach would also boost the area of xenotransplantation, which needs a solution of that level, representing a great improvement for biotechnological medicine as well.

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4.3 Patente - Recipiente térmico para transporte de ovários

Patente depositada no INPI (Instituto Nacional da Propriedade Industrial)

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

27/12/2019 870190140137 13:51

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2019 028010 7

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PETICIONAMENTO ELETRÔNICO

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	Recipiente térmico para transporte de ovários
Resumo:	A presente invenção refere-se a um recipiente térmico utilizado para transporte e armazenamento de ovários, com o intuito de suprir uma carência existente no estado da técnica. Para o sucesso da biotécnica de produção in vitro de embriões (PIVE), a qualidade dos oócitos é um fator limitante. Tais estruturas localizam-se nos ovários e são altamente sensíveis, principalmente a alterações de temperatura e à falta de suprimento sanguíneo. A fim de garantir o êxito da PIVE, a manutenção da temperatura e a agilidade no transporte dos ovários é essencial. Assim, o recipiente proposto possui uma bipartição com dois orifícios independentes, sendo um para a reserva de água e o outro para o armazenamento dos ovários. Além disso, é composto por duas tampas designadas para vedação dos compartimentos e contém dois suportes para alças removíveis, a fim de facilitar o processo de coleta e a manipulação do recipiente. Dessa forma, a presente invenção possui como objetivo final garantir a manutenção da temperatura para o transporte de ovários, garantindo sua qualidade além de ser de facil utilização e de se adequar ergonomicamente ao usuário através de sua disposição inovadora.
Figura a publicar:	1

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Resumo	Resumo.pdf
Procuração	PROCURAÇÃO_2017.pdf
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Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 27/12/2019 às 13:51, Petição 870190140137

5 CONCLUSÃO GERAL

Após uma robusta revisão bibliográfica, pode-se inferir que os principais protocolos de clonagem de suínos utilizados atualmente são a Transferência Nuclear de Células Somáticas (TNCS) e a Handmade Cloning (HMC), nos quais oócitos em metáfase II (MII) têm sido a principal escolha de célula receptora, bem como fibroblastos fetais e adultos vem sendo amplamente utilizados como células doadoras de núcleo. Estratégias de reprogramação nuclear como a inativação do cromossomo X, acetilação de histonas, metilação do DNA e remodelação da cromatina, vem sendo utilizadas para melhorar os resultados da clonagem. Além disso, a transferência do embrião clonado pode ser realizada em estágios iniciais (1 ou 2 dias após CIV) ou em estágio de blastocisto. Por fim, a estratégia de reclonagem, que possui um grande potencial que ainda não foi completamente explorado, pode ser utilizada para manutenção desse clone.

Ademais, levando em consideração que a reprogramação inadequada do genoma doador está intrinsicamente associada à baixa eficiência da clonagem, e que o oócito/citoplasma receptor executa papel fundamental nesse processo, foi realizado o depósito de patente de invenção de um recipiente capaz de manter os ovários na temperatura correta por um maior tempo durante as etapas de coleta, armazenamento e transporte, garantindo assim estruturas oocitárias com maior qualidade. Assim, pode-se apontar que a hipótese de que a produção de clones animais geneticamente editados possui limitações que podem ser supridas através de produtos ou processos inovadores é verdadeira.

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