The future is coming! New technologies being applied to reproduction in animals and humans

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Abstract

Advances in genome sequencing, gene editing and computational analyses have brought the genetic future rushing toward us. Gene editing is currently being used extensively in research, to explore function of genes via over- and under-expression, and to produce animal disease models and animals useful for production of medically important components. Through computational analysis of nucleotide sequence versus performance, disease or other morphological characteristics, tens of thousands of genetic variants associated with a large number of traits have been identified in many species, and selection based on genetic merit, even in preimplantation embryos, is ongoing. Advances are also being made in understanding and manipulation of primordial germ cell and oocyte development, such that functional oocytes have been produced from somatic cells; and in extended in vitro culture of embryos past the implantation stage. Combined with work on development of an artificial womb, the potential to achieve extra corporeal pregnancy seems plausible. Many of these advances appear to warrant veterinary, breed registration or ethical oversight, so it is important that veterinarians be aware of new achievements in these areas.

Keywords: Oocyte, embryo, stem cells, gene editing, uterus

Gene editing

Claudia Klein at the 2019 Society for Theriogenology Annual Conference presented an in-depth review of methods for gene editing and provided useful references regarding these techniques.[1] Gene editing methods have come to the forefront; most useful are **TALENs** (Transcription Activator-like Effector Nucleases) and **CRISPR** (Clustered Regularly Interspaced Short Palindromic Repeats).

TALENs are proteins. They recognize specific sections of DNA via similar mechanisms to those that transcription factors use: the protein structure of the TALEN binds only to a specific nucleotide sequence. TALENs are linked to effector proteins.

CRISPERs are RNA nucleotide sequences. One section of the CRISPER is designed to be complementary to the target DNA nucleotide sequence and so recognizes and binds to that specific section of DNA; the second section of the CRISPR molecule is associated with an effector protein.

Both TALENs and CRISPRs work via the **effector proteins** associated with them. For gene editing, the linked effector protein is an endonuclease (an enzyme that cuts DNA); the specific endonuclease CAS9 (CRISPR-Associated protein 9) is typically linked to CRISPR. When a TALEN or CRISPR is bound to the recognized nucleotide sequence, the endonuclease will cut the DNA at a specific nucleotide, adjacent to the location of the TALEN or CRISPR. Since the first use of guided endonucleases to induce site-specific DNA breaks,[2-4] many other kinds of effector proteins have been linked to these targeting molecules: proteins that cause a gene to "turn on" and start transcription; proteins that inhibit transcription and even proteins that deliver epigenetic markers (reviewed[5]). Linkage of these proteins to targeting molecules that recognize specific nucleotide sequences allows the investigator to modify the DNA sequence, gene activity, or epigenetic profile of targeted areas of genome.

The most straightforward use of gene editing tools is to create a mutation that causes a gene to be inactive. For this, editing tools are designed to make a double-stranded cut in DNA, which the cell finds difficult to repair while maintaining fidelity. Double-stranded repair thus is typically associated with changes to the nucleotide sequence resulting in reading frame shifts in DNA, and thus in a mutation that can completely inactivate gene.

It is also possible to change the sequence of a gene in a directed way, by making a break and providing the cell with multiple copies of a template, i.e. pieces of DNA with the desired nucleotide sequence. The cell uses the template to direct repair of the cut section, resulting in the desired genome

sequence in some cells. Remarkably, it is not necessary in all cases to provide a template; if the homologous chromosome has a different sequence for the targeted gene (which thus is not affected by the editing tool), mammalian cells can use the homologous chromosome as a template to fix a double strand break.[6] In this way, cells that are heterozygous for an undesired mutation can "fix" the gene sequence in mutated gene, without introduction of foreign DNA.

Gene editing tools can be used to produce animals with a desired genetic modification in 2 main ways: 1) by editing somatic cells, selecting a cell with the desired modification, and performing somatic cell nuclear transfer (SCNT); or 2) by injecting the editing tool into a zygote or early embryo, thereby directly editing the genome of the zygote/embryo and thus all resulting embryonic cells. A third area of intense interest is using these tools to correct disease-related genetic mutations in living animals (gene therapy).

Gene editing has been used in almost all domestic species, including horses (embryos with knockout for myostatin, produced by SCNT)[7] and dogs (gene therapy to correct the muscular dystrophy gene;[8] production of myostatin-knockout dogs via zygote injection).[9] It has also been applied to a wide variety of nondomestic animals, including macaques, ferrets, koi, zebrafish, squid, shrimp beetles, butterflies, and bees, to study genetic factors related to their behavior and health.[10,11] The ethics of gene editing to "fix" mutated genes in human embryos is currently being debated.

Problems with POLLED gene-edited calves

Stories involving gene editing have recently been in the news. An American company, Recombinetics, published a report in 2016, announcing production of 2 crossbred dairy bulls produced via SCNT with somatic cells edited with TALENs, that were consequently homozygous for the POLLED allele.[12] The company termed this method "precision breeding" (http://recombinetics.com/naturally-hornless-cattle/). They proposed that this would be a good model for introduction of gene-edited livestock into food production, as progeny of these animals would not require debudding to remove horns, a procedure with animal welfare concerns. However, genome sequencing of the gene-edited bulls' progeny(University of California, Davis) revealed a bacterial antibiotic-resistance gene in some calves' DNA (apparently, transferred to cells during the editing process) that was not detected in the original testing of the bulls.[13] These findings represent a dramatic setback to possibility of acceptance of gene-edited livestock for enhancement of production, both because of presence of "off-target" changes in the animals' DNA and because the problem was not revealed during initial genetic testing.

Birth of gene-edited human babies

In a second event regarding gene editing, a scientist in China, He Jiankui, announced via a video released on YouTube in November, 2018, the birth of human twins which he claimed had undergone gene editing as zygotes. The editing was to induce a genetic modification, Δ32 mutation in the CCR5 gene, which when naturally occurring confers resistance to entry of a common variant of HIV virus. This human experimentation violated Chinese regulations banning genome editing on human embryos, as well as basic scientific and ethical guidelines (https://www.nytimes.com/2018/12/05/health/crispr-gene-editing-embryos.html) and was condemned by scientists in China and abroad.[14] The Scientific Ethics Committee of Academic Divisions of the Chinese Academy of Sciences stated that "the theory is not reliable, the technology is deficient, the risks are uncontrollable, and ethics and regulations prohibit the action."[14] The gene-edited embryos were apparently biopsied to confirm presence of mutation, and then transferred, resulting in birth of twin girls. However, further evaluation of the process used revealed that the editing process was not precisely directed, and that both children have a mosaic of different mutations of the targeted gene, which may or may not confer resistance to HIV. He Jiankui was fired by his university, and in December 2019 was sentenced to 3 years in prison; the court found that this researcher and his collaborators forged ethical approvals and did not inform the physicians transferring the embryos of their origin.

Use of gene-edited mice and time-lapse photography to study embryo development

The study of embryo development has progressed markedly with the use of time-lapse, within-incubator photography, currently utilized extensively in human assisted reproductive technology (ART; reviewed[15]) and recently applied to clinical equine IVP embryos.[16-20] Work in mouse embryos has gone a step further: using the precise and efficient new gene editing tools, mice have been produced in which key cytoplasmic components were labeled with fluorescent markers. Combining these markers with fluorescent molecular labels and using time-lapse fluorescent microscopy, it is possible to visualize changes in cytoskeletal, nuclear and cell-lineage specific components during embryo development.[21] Using information gained in this manner, scientists are beginning to outline morphogenetic mechanisms involved in early embryo and blastocyst development.[22]

Embryo biopsy, testing and selection

Cattle

The availability of immense amounts of production data on bulls, cows, and their progeny has allowed identification of hundreds of thousands of genetic markers (single polymorphic nucleotides, SNPs); tens of thousands of which have been associated with desirable traits, both in beef and dairy cattle. Microarrays for rapid detection of production-related SNPs are marketed commercially and have been used since the mid 2000s[23] to select calves for genetic merit at birth, resulting in rapid and meaningful gains in productivity.[24] These genetic selection methods are now being applied to embryos, via cells derived from embryo biopsy, to allow selection of embryos before transfer.[25] The ability to select embryos with high genetic merit, combined with the ability to recover competent oocytes from gonadotropin-treated heifer calves as young as 2 months of age for in vitro embryo production,[26] are expected to result in greatly accelerated genetic gain through decreasing generation time.

Horses

Methods for effective biopsy and genetic analysis of both in vitro-produced and in vivo-derived equine embryos have been developed.[27,28] Currently, this procedure is used clinically, mainly for testing embryos for presence of disease-related mutations[29] or for fetal sex.[30] Because horses lack the immense database found in cattle, information on genetic markers for performance in horses is more difficult to generate; however, an increasing amount of information is available in this area. In a 2010 report, variants of myostatin gene, apparent as SNPs, were found to be strongly associated with success of horses at different racing distances.[31] Subsequently, equine SNPs were identified that are associated with "gaitedness", e.g. found in horses that deviate from standard two-beat trot and instead perform running walk, rack, singlefoot, etc.;[32] with size, e.g. miniature, pony, horse, draft horse;[33] breed type;[34] and with selection for speed in Australian thoroughbreds, via SNPs in a chromosome location associated with neuromuscular junction signaling.[35] Because effective methods for embryo biopsy are being developed in horses, the potential for preimplantation genetic selection in horses, as done in cattle, is growing..

Humans

Preimplantation genetic testing (PGT) has been performed on in vitro-produced embryos since the 1990's in couples known to carry disease-related mutations.[36] The term PGT refers to evaluating genetics of cells recovered on biopsy of embryo (embryonic blastomere of an early-cleavage embryo -- no longer used because of deleterious effects on embryo -- or trophoblast of a blastocyst). PGT can be divided into several types of assessments. Testing for a specific allele, typically 1 carrying a disease-related mutation (recently re-termed PGT-M; M for monogenic) was developed first. Subsequently, methods to evaluate the entire genome of cells obtained by biopsy were introduced, to determine aneuploidy (duplication or deletion of chromosome segments or entire chromosomes). Such genome-wide screening is currently termed PGT-A (A for aneuploidies). This screening was initially done by comparative genomic hybridization and is now done via genome sequencing. The jury is still out as to whether employing PGT-A to screen embryos before transfer actually increases the chances of live birth,

as there can be aneuploidies in the trophoblast that are not reflected in the embryo proper and the time needed for results to be available precludes fresh transfer of embryos.[37]

The ability to evaluate the entire genome of embryos, however, has led to the possibility of selecting embryos for desired traits. Gender selection of human embryos (without a medical reason such as to avoid an X-linked disease) is illegal in some countries, but is legal, and offered, in other countries, including the US. Human ART labs are already offering selection for eye color and possibly other characteristics, and methods to select other traits, including intelligence (selection against genes known to be associated with a low IQ), have been developed and companies plan to market them (https://genomicprediction.com/). Human ART centers may soon be calculating a polygenic "risk score" for each embryo, based on known genetic markers of disease susceptibility, to allow selection of embryos likely to produce the healthiest individuals.

Recent advances with application to species conservation

Major barriers for SCNT use in conservation of endangered species are: 1) availability of oocytes from appropriate species, i.e. oocytes having species-specific mitochondria and cytoplasm compatibility with species' genome, to use as host oocytes for SCNT; and 2) availability of a suitable recipient female – that is, a female that is fertile, cyclically synchronous with the embryo, capable of gestating the transferred embryo and in which embryo transfer can be performed (for example, there is no established method to transfer embryos to the rhinoceros uterus). However, several recent advances have potential for application in this area:

Production of functional oocytes from somatic cells

This possibility in mice was reported in 2012.[38] Somatic cells were first induced to be pluripotent by inserting genes coding for pluripotency factors, then these induced pluripotent stem cells (iPSCs) were caused to differentiate to primordial germ cell-like cells (PGCs). These were aggregated with embryonic ovarian cells and transplanted to the ovarian bursa of recipient females. The somatic-origin cells differentiated to oocytes and were recovered from the recipient ovary at the germinal vesicle stage and matured in vitro, fertilized in vitro and yielded live young.

In 2019, production of oocytes from somatic cells (granulosa cells) **without transgenesis** (using only chemical stimulation), using a similar series of procedures, was reported.[39] This is a huge leap forward toward eventual use of somatic cells for derivation of species-specific oocytes, even from totally extinct species. Many zoological parks have tissue or fibroblast samples from numerous species stored in liquid nitrogen; these somatic cells could be used for production of species-specific oocytes. Lack of embryonic ovarian cells from desired species to use for aggregating with somatic-origin PGCs should not be a barrier, since aggregation of oocytes, recovered from primordial follicles, with ovarian cells of other species is compatible with production of functional oocytes.[40]

The prospect of deriving oocytes from somatic cells in this manner has great application in assisted reproduction to aid conservation of endangered species. Mitochondrial-identical oocytes could be used as host oocytes for somatic cell nuclear transfer, to produce clones using stored tissue samples. Even more exciting is prospect of such oocytes could be fertilized using stored sperm from males selected to produce offspring with greatest genetic diversity possible. In this manner, the population genetic diversity could be maximized and the genetics of animals for which only sperm is available could be reintroduced into the population.

Production of embryo-like structures directly from stem cells

The term "totipotent" is used to refer either to a cell capable of developing into a complete organism (e.g. a fertilized oocyte), or to a cell that can differentiate into any cell type of an organism. However, it was felt that these are 2 different issues.[41] An oocyte can develop into entire organism after fertilization or nuclear transfer, whereas an embryonic stem cell (capable of contributing to any tissue of organism) cannot generate and organize entire organism. The term "plenipotent" has been proposed for this second, less capable cell.[41]

However, recent work has begun to blur these lines. Production of embryo-like structures from aggregated human stem cells is possible.[42] Through a gel-based 3-D environment created by a microfluidic device, stem cells were induced to recapitulate early aspects of epiblast and amnion development, including cell line differentiation, lumen formation, polarity in the embryonic sac, specification of primordial germ cells and, notably, cells with primitive streak markers.

Derivation of induced pluripotent stem cells from somatic cells has been reported in several endangered species, including mandrill and northern white rhinoceros.[43] Thus, in the future, embryos might be derived directly from pluripotent stem cells. This opens the door to use of stored somatic cells to produce mitochondrial-identical clones of endangered and even extinct species without need for oocytes, or for going through the laborious steps of deriving primordial germ cells and then oogonia from stem cells.

Culturing embryos in vitro past blastocyst stage

Monkey embryos have been cultured in vitro for up to 20 days.[44] Embryos underwent differentiation to developmentally-appropriate cell lines and stages, including embryonic disc formation, amniotic and yolk sac cavitation, and differentiation of primordial germ cell-like cells. Human embryos have also been cultured in vitro past 7 days, with cell lineage differentiation, but culture was terminated at 14 days for ethical reasons.[45,46] The ability to culture embryos in vitro to the point of differentiation and cell lineage specification is both unsettling, in terms of producing fetuses "in a test tube" and promising, in terms of being able to propagate embryos from endangered species without the need for a suitable recipient female. It appears that research will continue to push the ability to support embryonic development in vitro further and further into pregnancy.

Artificial womb

An in vitro method to support development of lambs during last 4 weeks of pregnancy has been developed.[47] Lambs were removed from the uterus and placed in a sterile polyethylene bag that was cannulated to allow continuous fluid exchange in an artificial "amnion," and an oxygenating circuit was connected to the fetal umbilical cord. Extensive work was performed to optimize pressures, circuit flows, oxygenation and other mechanical aspects to reproduce the environment of the gravid uterus and support normal development of the lamb. With the optimized system, lambs at a stage equivalent to extreme prematurity in human infants survived for 4 weeks, had stable hemodynamics and normal growth, including lung development and brain maturation. Lambs were removed, ventilated and then euthanized to assess tissue development.

The potential application of this technique in ART for endangered species is immense. As in vitro early embryo culture methods continue to progress further toward supporting embryo development through the placental and fetal stages, perhaps these methods for supporting the fetus after organogenesis and umbilical cord formation will be applicable earlier and earlier in pregnancy, with the eventual possibility of supporting the entire pregnancy of an embryo/fetus without a species-compatible recipient female.

Conclusion

In many ways, science is continuing to explore techniques proposed for decades by science-fiction writers in an imagined future. Embryo production in vitro is commonplace; genetic selection is being used widely in cattle and is also utilized in human embryos, currently largely to avoid transferring embryos with devastating genetic diseases. Gene editing is being performed for research but has much interest from commercial sector, including human ART. It is difficult to predict future applications of these approaches; the ethical lines that society draws in these areas appear to move, as past innovative technology becomes commonplace and accepted.

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