ADVANCES IN THE PRODUCTION AND PROPAGATION OF TRANSGENIC GOATS USING LAPAROSCOPIC OVUM PICK-UP AND IN VITRO EMBRYO PRODUCTION TECHNOLOGIES

H. Baldassarre, B. Wang, N. Kafidi, C. Keefer, A. Lazaris, and C.N. Karatzas

Nexia Biotechnologies Inc., Vaudreuil, Quebec, Canada

ABSTRACT

Laparoscopic ovum pick-up (LOPU) is a convenient methodology by which oocytes can be recovered and used either for in vitro production of zygotes or as a source of cytoplasts in nuclear transfer (NT) procedures. The pregnancy and transgenesis rates achieved with IVM/IVF of LOPU-sourced oocytes followed by subsequent DNA microinjection of zygotes are similar to the rates obtained when using in vivo-produced oocytes or zygotes. Similarly, pregnancy rates and kids born by using LOPU-sourced and in vitro matured oocytes as recipient cytoplasts in NT programs are comparable with those reported by others using in vivo matured oocytes collected by oviduct flushing. The use of LOPU allows for improved control over the stage of maturation/development of the oocytes and produced zygotes, a less invasive means of recovery, thereby allowing for repeated usage of the oocyte donor animals and the ability to source the oocytes from live animals of known health status. In addition, because of large follicular responses that can be obtained from prepubertal animals, LOPU followed by IVM/IVF has demonstrated great potential for the early propagation of valuable animals, in particular, transgenic animals.

INTRODUCTION

The production of transgenic animals has been thoroughly reviewed by others (22, 23, 30, 31). Because of the outstanding protein synthetic capacity of the mammary gland, transgenic animals are able to produce recombinant proteins in a more efficient manner than traditional systems based on microorganisms or animal cells. This economic opportunity has stimulated the development of a new industry, as well as new applications for embryo production technologies.

In the system we have established, immature oocytes collected by LOPU is the starting point for the production and early propagation of transgenic goats. The LOPU technique is very reliable and allows for the recovery of a predictable number of oocytes during each session. It is also less invasive than standard surgery (laparotomy) used for the recovery of in vivo zygotes and in vivo matured oocytes, thus allowing multiple recoveries from the same donor animal.

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Unlike oocytes collected from slaughterhouse ovaries, LOPU-derived oocytes are recovered from animals of known health status. The health status of transgenic animals producing therapeutic proteins for pharmaceutical applications is of vital importance for the regulatory approval process of specific products.

**LOPU**

Snyder and Dukelow first described the recovery of 6 oocytes from a sheep by follicular aspiration of 21 follicles under laparoscopic observation in 1974. However, the technique was not fully developed until recent years, in conjunction with the development of in vitro embryo production technologies (2, 3, 6, 15, 16, 28, 29).

Laparoscopic ovum pick-up is of major importance in the production and early propagation of transgenic goats. Following IVM/IVF of LOPU-sourced oocytes, the resulting zygotes can be microinjected with a DNA construct of interest for the generation of transgenic founder animals. As an alternate approach for the production of transgenic goats, in vitro matured oocytes can be fused to transgenic donor cells by use of the NT technique. Furthermore, LOPU-derived oocytes can be used in IVP procedures for early propagation of transgenic founders of both genders.

The LOPU procedure is described in detail elsewhere (2). Briefly, donor goats are restrained on a standard laparoscopy table under general anesthesia, and follicles are aspirated under laparoscopic observation using a 20-gauge needle mounted in a plastic pipette connected to a collection tube and vacuum line.

To recover high numbers of oocytes per LOPU session, the donor goats are stimulated with gonadotrophins. Different hormonal regimes were tested during the technique setup using adult donors of the Nigerian Dwarf breed (BELE®, Breed Early Lactate Early). Based on previous work reported in sheep (3), an FSH multi-injection regime was compared with a combination of FSH and eCG in an “oneshot” regime. Goats were synchronized by means of intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix®, Upjohn, Canada) inserted 10 d prior to LOPU and an injection of 125 µg Estrumate® (Malinkrodt, Canada) on the morning of the 8th day. Sponges were removed at the time of LOPU. In the multi-injection regime, the goats received a total equivalent to 133 mg NIH-FSH-P1 of Folltropin®-V (Vetrepharm, Canada) given twice daily over the 48 h prior to LOPU. In the “oneshot” regime, goats were injected with 80 NIH-FSH-P1 of Folltropin®-V and 300 IU eCG (Equinex®, Ayerst, Canada) in a single application at either 36 or 48 h prior to LOPU. Results are shown in Table 1.

Table 1. Follicles aspirated and oocytes recovered (average ± sd) from BELE® (Breed Early Lactate Early) goats treated with multiple FSH injections vs. “oneshot” hormonal regimes.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Treatment</th>
<th>n</th>
<th>Follicles</th>
<th>Oocytes</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BELE®</td>
<td>FSH multiple</td>
<td>12</td>
<td>14.0 ± 5.3</td>
<td>12.7 ± 5.6</td>
<td>90.71</td>
</tr>
<tr>
<td>BELE®</td>
<td>Oneshot 36</td>
<td>15</td>
<td>11.2 ± 5.0</td>
<td>10.0 ± 5.7</td>
<td>89.29</td>
</tr>
<tr>
<td>BELE®</td>
<td>Oneshot 48</td>
<td>14</td>
<td>11.7 ± 5.4</td>
<td>10.1 ± 5.6</td>
<td>86.32</td>
</tr>
</tbody>
</table>

Results in the same column are not different (ANOVA, P >0.05).
The effect of the "oneshot" regime applied 36 or 48 h prior to LOPU in adult donors of standard breeds (Alpine and Saanens) is shown in Table 2.

Table 2. Number of follicles aspirated and oocytes recovered by LOPU (laparoscopic ovum pick-up) of goats of standard breeds (average ± sd) treated with the "oneshot" regime 36 or 48 h prior to LOPU.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Treatment</th>
<th>n</th>
<th>Follicles</th>
<th>Oocytes</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Oneshot 36</td>
<td>17</td>
<td>27.5 ± 10.8</td>
<td>24.0 ± 10.7</td>
<td>87.27</td>
</tr>
<tr>
<td>Standard</td>
<td>Oneshot 48</td>
<td>14</td>
<td>24.4 ± 8.9</td>
<td>20.1 ± 6.9</td>
<td>82.38</td>
</tr>
</tbody>
</table>

Results in the same column are not different (ANOVA, P>0.05).

Although the "oneshot" regime is preferred over the multiple injection regime for its simplicity, injection 36 h prior to LOPU appeared to be a more appropriate timing for the oneshot regime, as it resulted in fewer undesired ovulations at the time of LOPU. In addition, the administration of hormones 48 h before LOPU resulted in more follicles showing thick follicular content, which were more difficult to aspirate, as well as a larger number of oocytes with expanded cumulus (data not shown).

Of particular interest is the outstanding follicular response obtained from gonadotrophin-stimulated prepubertal animals (Table 3). Such exceptional responses were first described in cattle (1) and sheep (10, 11). We have recently described similar results in goats (20).

Table 3: Follicles aspirated and oocytes recovered by LOPU (laparoscopic ovum pick-up) (average ± sd) from adult vs. prepubertal (3 to 5 months of age) donor goats of standard breeds (Koeman et al., Theriogenology, 2000).

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Follicles</th>
<th>Oocytes</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepubertal</td>
<td>23</td>
<td>39 ± 4.5a</td>
<td>28.4 ± 3.5a</td>
<td>73%</td>
</tr>
<tr>
<td>Adult</td>
<td>21</td>
<td>19 ± 1.4b</td>
<td>15.9 ± 1.5b</td>
<td>84%</td>
</tr>
</tbody>
</table>

Values in the same column with different superscript are different (ANOVA, P <0.01).

The hormonal regime used in prepubertal animals was the same "oneshot" regime used for adults without the use of the sponge and the Estrumate® injection, as heat synchronization is not necessary. In agreement with data reported in sheep (11), our studies have demonstrated that significantly pronounced follicular response and higher oocyte yields are recovered by LOPU from stimulated prepubertal goats at less than 3 months of age (Table 4).
Table 4. Follicles aspirated and oocytes recovered by LOPU (laparoscopic ovum pick-up) (average ± sd) from prepubertal goats of standard breeds at two different age ranges at the time of first collection.

<table>
<thead>
<tr>
<th>Age range</th>
<th>n</th>
<th>Average age</th>
<th>Follicles</th>
<th>Oocytes</th>
<th>Recovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-90 d</td>
<td>20</td>
<td>74 ± 7 d</td>
<td>59.3 ± 28 *</td>
<td>49.7 ± 24 *</td>
<td>84%</td>
</tr>
<tr>
<td>90-150 d</td>
<td>36</td>
<td>116 ± 15 d</td>
<td>34.4 ± 20 b</td>
<td>27.4 ± 14 b</td>
<td>80%</td>
</tr>
</tbody>
</table>

Values in the same column with different superscript are different (ANOVA, \( P < 0.001 \)).

In agreement with previous reports in sheep (11, 21, 24), acceptable development of these oocytes aspirated from prepubertal goats can be obtained following IVM and IVF procedures. Of 637 oocytes recovered from 28 prepubertal donors, 365 (57%) were at the 2 pronuclei stage 20 h after IVM/IVF. Thirty zygotes transferred into 4 recipients resulted in 2 pregnancies and 3 kids born. Nine additional pregnancies resulting from 139 zygotes transferred into 23 recipients are in utero. This information is very valuable for the early propagation of transgenic female founder goats.

PRODUCTION OF TRANSGENIC GOATS USING LOPU-SOURCED OOCYTES AND ZYGOTE PRONUCLEAR MICROINJECTION FOLLOWING IVM/IVF

In vitro-produced zygotes from LOPU-sourced oocytes have been DNA microinjected to produce transgenic goats (Baldassarre et al, manuscript in preparation). In vitro maturation and IVF were performed using standard procedures (20, 33). The IVM was carried out in 50-μL drops of TCM 199 supplemented with hormones and 10% heat-inactivated estrus goat serum at 39°C in a humidified incubator with 5% CO₂ in air for 24 to 27 h. The IVF was performed in 50-μL drops of TALP medium supplemented with 20% heat-inactivated estrus goat serum at 39°C in a humidified incubator with 5% CO₂ in air. The fertilization drops were inseminated with pre-capacitated fresh semen at a final concentration of \( 1 \times 10^6 \) sperm/mL. After co-culture of the gametes for 15 to 20 h, the presumptive zygotes were microcentrifuged to improve pronuclear visualization, and those with a visible pronucleus were microinjected with the designated DNA construct. Successfully microinjected zygotes were then transferred into the oviduct of recipient goats by mid laparotomy using a Tomcat® catheter (Sovereign, Canada).

The results obtained by DNA microinjection of in vitro zygotes produced by IVM/IVF of LOPU-sourced oocytes are summarized here.

<table>
<thead>
<tr>
<th>Donors aspirated</th>
<th>210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicles aspirated (average per goat)</td>
<td>3293 (15.7 ± 9)</td>
</tr>
<tr>
<td>Oocytes recovered (average per goat)</td>
<td>2823 (13.4 ± 8)</td>
</tr>
<tr>
<td>Zygotes microinjected</td>
<td>1482</td>
</tr>
<tr>
<td>Zygotes transferred</td>
<td>1366</td>
</tr>
<tr>
<td>Recipients transferred (average embryos per recipient)</td>
<td>219 (6.2)</td>
</tr>
<tr>
<td>Recipients pregnant at 30-d ultrasound (% pregnant)</td>
<td>109 (50%)</td>
</tr>
<tr>
<td>Kids born</td>
<td>150</td>
</tr>
<tr>
<td>Kids transgenic (% of kids born)</td>
<td>9 (6%)</td>
</tr>
<tr>
<td>Transgenic kids of zygotes microinjected</td>
<td>0.6%</td>
</tr>
</tbody>
</table>
The transgenesis rates are comparable with those reported by us (4) and others (13) obtained by using in vivo produced goat zygotes.

PRODUCTION OF TRANSGENIC GOATS USING LOPU-DERIVED OOCYTES AND NT TECHNOLOGY

Nuclear transfer has the potential to increase the efficiencies of producing transgenic animals over traditional methods such as pronuclear microinjection (9, 22). Donor cells to be used as donor nuclei in NT can be transfected in vitro with the DNA construct of interest and tested for integration before they are fused to an enucleated oocyte and transferred to a recipient (18). As a result, fewer recipients are needed to produce the same number of transgenic animals when compared with pronuclear microinjection. Furthermore, all of the animals are of the desired gender, making the system more efficient than the traditional pronuclear microinjection technology.

Although in vivo matured oocytes have been used most commonly as recipient cytoplasts for NT programs in sheep (32, 25) and goats (7), our system is based on the use of in vitro matured oocytes collected by LOPU. This allows us to avoid surgical collection of oocytes (by laparotomy) and have better control over the stage of maturation of the oocytes.

Fetal fibroblasts isolated from 27 to 30-d-old fetuses were transfected with the DNA constructs of interest. Stable transfectants were selected using G418 treatment. Donor cells were cultured in low serum (0.5% FCS) for 4 d prior to use in NT. Procedures for enucleation, fusion, and activation have been described elsewhere (18).

Results obtained by NT using transfected fetal fibroblasts and in vitro matured oocytes are shown below (Keefer et al., 2002).

| LOPU-derived oocytes/IVM: | 1700 |
| Embryos transferred (% of oocytes) | 830 (49%) |
| Recipients pregnant at 30 d (%) | 21/74 (28%) |
| Recipients to term (%) | 16 (76%) |
| Kids born | 26 |
| Survived (%) | 17 (65%) |

The kids surviving represent 1% of the total in vitro matured oocytes and 2% of the reconstructed embryos transferred. These results are comparable with the results published by others working with in vivo matured oocytes in sheep (25) and goats (7).

PROPAGATION OF TRANSGENIC FOUNDER GOATS USING LOPU-SOURCED OOCYTES AND IVP

Transgenic female founder goats can be hormonally induced to lactate at 60 to 90 d of age to test for the presence and levels of the recombinant protein of interest. Animals expressing the recombinant protein of interest at the desired levels represent valuable animals, and it is desirable to propagate
their genetics as early as possible. Laparoscopic ovum pick-up in combination with embryo production technologies can be used for that purpose at two levels.

On one hand, shortly after confirming expression of the desired re-protein in milk, female goats are hormonally treated and subjected to LOPU. The collected oocytes undergo IVM/IVF, and the resulting embryos are transferred to recipients, resulting in kids born at the time the donors are reaching breeding age. This shortens the timelines for producing offspring of valuable female founders by at least 5 months. In addition, this method has the potential to be very efficient in terms of the number of kids born, as it takes advantage of the high numbers of oocytes collected from stimulated prepubertal animals.

On the other hand, the cumulus-granulosa cells obtained during LOPU of these transgenic animals can be used for their propagation via NT (Keefer et al., 2002).

Similarly, the production of offspring from transgenic founder male goats can be accelerated by means of using IVP technologies. Semen samples can be obtained as early as 15 wks of age (14) and used for IVF of LOPU-sourced oocytes 4 to 5 months before the male will be capable of producing enough semen to perform an AI program.

The following results corresponds to the in vitro production of embryos using semen collected from a 5-mo-old transgenic buck from our herd.

<table>
<thead>
<tr>
<th>Oocytes</th>
<th>Fertilized</th>
<th>Recipients</th>
<th>Pregnant</th>
<th>Kids born</th>
<th>Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>242</td>
<td>129</td>
<td>21</td>
<td>14 (67%)</td>
<td>17</td>
<td>6 (35%)</td>
</tr>
</tbody>
</table>

In a similar fashion semen from transgenic bucks can be used to fertilize in vitro oocytes sourced by LOPU from transgenic goats.

POTENTIAL APPLICATIONS OF LOPU ON SMALL RUMINANT IMPROVEMENT PROGRAMS

Repeated collection of oocytes followed by IVM/IVF/IVC has the potential for producing more offspring from genetically valuable goats than traditional multiple ovulation and embryo transfer (MOET) procedures (29). Thanks to its less invasive nature, the procedure can be repeated more times than laparotomy, traditionally used for embryo recovery in small ruminants (27). In addition, it is an interesting approach to overcome problems of early regression of corpora lutea, that is a cause of failure in up to 30% of the donors submitted to MOET. It also provides the possibility of producing offspring in situations where MOET is not applicable, such as in pre-pubertal, gestating, puerperal, and aged animals.
From an economic point of view, the most attractive technology may be the in vitro production of embryos from oocytes recovered from prepubertal goats. The high number of oocytes recovered from young animals combined with an acceptable developmental capacity has the potential to significantly shorten the generation interval, hence accelerate the rate of genetic progress. In traditional breeding programs, animals of proven genetic merit and production traits are selected for breeding. However, this information is usually not available in prepubertal animals. Laparoscopic ovum pick-up of prepubertal animals can accelerate the process of expansion of a limited number of genetically valuable animals when introduced in a new region.

Goat production traits such as modification of milk composition, increased growth rate, or the improvement of mohair fiber production may benefit from transgenic technology. However, for the technology to find widespread application in the improvement of such production traits, the genes that influence the specific traits need to be identified, efficiencies need to be improved, and, equally as important, the cost of generating and proving the genetic gain should be decreased.

Similarly cloning has the potential to improve the efficiency of transgenesis in the applications just mentioned, as well as a role for the multiplication of animals of proven production. However, issues of low pregnancy and survival rate and lack of genetic diversity need to be resolved before it becomes part of the livestock improvement strategies.

CONCLUSIONS

Laparoscopic ovum pick-up is an efficient and reliable method to obtain immature oocytes to be used in the production and propagation of transgenic goats in combination with embryo in vitro production technologies.

Pronuclear microinjection of zygotes following IVM/IVF of LOPU-sourced oocytes results in comparable transgenesis rates with those obtained when using in vivo produced zygotes.

Similarly, pregnancy rates and goat kids born using in vitro matured oocytes obtained by LOPU as recipient cytoplasts in a NT program are comparable with those reported in the literature when using in vivo matured oocytes collected by oviduct flushing.

The main advantages of the LOPU methodology over the utilization of in vivo produced zygotes or in vivo matured oocytes are improved control over the stage of maturation/development of zygotes and oocytes and the less invasive means of recovery, thereby allowing for repeating the LOPU procedure to the donor animals several times. In addition, compared with oocytes sourced form abattoir ovaries, LOPU allows one to source the oocytes from live animals of known health status, which is of vital importance for the production of therapeutic proteins for pharmaceutical applications.
Finally, because of the pronounced follicular responses that are observed in stimulated prepubertal animals, LOPU followed by IVM/IVF may prove beneficial for the early propagation of transgenic animals.

REFERENCES


