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Non-viral transfection of goat germline stem cells by nucleofection results in production of transgenic sperm after germ cell transplantation

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Abstract

Germline stem cells (GSCs) can be used for large-animal transgenesis, in which GSCs that are genetically manipulated in vitro are transplanted into a recipient testis to generate donor-derived transgenic sperm. The objectives of this study were to explore a non-viral approach for transgene delivery into goat GSCs and to investigate the efficiency of nucleofection in producing transgenic sperm. Four recipient goats received fractionated irradiation at 8 weeks of age to deplete endogenous GSCs. Germ-cell transplantations were performed 8-9 weeks post-irradiation. Donor cells were collected from testes of 9 week-old goats, enriched for GSCs by Stapt velocity sedimentation, and transfected by nucleofection with a transgene construct harboring the human growth hormone gene under the control of the goat beta-casein promoter (GBC) and a chicken beta-globin insulator (CBGI) sequence upstream of the promoter. For each recipient, transfected cells from 10 nucleofection reactions were pooled, mixed with non-transfected cells to a total of 1.5×10^8 cells in 3ml, and transplanted into one testis ($n = 4$ recipients) by ultrasound-guided cannulation of the rete testis. The second testis of each recipient was removed. Semen was collected starting at 9 months after transplantation for a period of over a year (a total of 62 ejaculates from 4 recipients). Nested genomic PCR for hGH and CBGI sequences demonstrated that $31.3\% \pm 12.6\%$ of ejaculates were positive for both hGH and CBGI. This study provides proof-of-concept that non-viral transfection (nucleofection) of primary goat germ cells followed by germ cell transplantation results in transgene transmission to sperm in recipient goats.

Keywords

Transgenic animals; germline stem cells; spermatogenesis; nucleofection; germ cell transplantation

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Introduction

Male germline stem cells (GSCs) are unipotent stem cells in the testis that self-renew and undergo differentiation to form sperm (Dym 1994). The ability of GSCs to transmit genetic information to the next generation makes them an attractive target for transgene transmission. Harnessing their power as a transgene carrier, however, has initially been a daunting task as GSCs are scarce and difficult to access in vivo. The first breakthrough came in 1994, when Brinster and coworkers developed the technique of germ cell transplantation (Brinster and Zimmermann 1994). They discovered that GSCs from a donor mouse testis, when transplanted into the seminiferous tubules of an infertile recipient testis, were able to colonize the recipient testis and re-established long-term donor-derived spermatogenesis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). These studies laid the foundation for new technologies in transgenic research because they demonstrated that GSCs can be genetically manipulated in vitro, and then transplanted into a recipient testis to generate transgenic gametes. The first attempt to generate transgenic animals using GSC transplantation was performed by retroviral transduction of mouse GSCs in vitro, followed by transplantation into recipient testes (Nagano et al. 2000). Approximately 4.5% of progeny sired by recipients were transgenic, and the transgene was passed on to subsequent generations (Nagano et al. 2001). Since then, other viral vectors, such as lentiviral, adenoviral, and adeno-associated viral vectors have been used for GSC transduction (Honaramooz et al. 2008; Nagano et al. 2002; Takehashi et al. 2007). The same concept was also successfully applied for the production of transgenic rats through viral transduction (Hamra et al. 2002; Ryu et al. 2007), and recently transposon-mediated transduction of rat GSCs (Izsvak et al. 2010).

Transgenic large animal models are important in biomedical research, agriculture and biopharmaceutical production. For large animals in which embryonic stem cells are not yet widely available, the prevailing approaches for generating transgenic animals are pronuclear microinjection or somatic cell nuclear transfer (SCNT) (Gama Sosa et al. 2010; Piedrahita and Olby 2011). Those approaches have relatively low efficiency, and animals resulting from SCNT frequently suffer from placental abnormalities (Bacci 2007; Dinnyes et al. 2008). We previously demonstrated that GSCs of goats are capable of transgene transmission after adeno-associated virus (AAV)-mediated transduction and subsequent transplantation into the testes (Honaramooz et al. 2008). This research lent support to the notion that transgenesis through GSCs can be useful for large animal transgenesis.

Although viral vectors are suitable for transducing slowly dividing GSCs, the concerns for safety have prompted searches for alternative gene delivery methods. In this study, we explored a non-viral approach (nucleofection) to introduce a transgene into goat GSCs. Nucleofection is an electroporation-based transfection method that utilizes a combination of electric pulses and optimized transfection reagents for transgene delivery (<http://www.lonzabio.com/cellbiology/transfection/technology/>). It was reported to deliver transgenes directly into the nucleus with high efficiency (Lorenz et al. 2004; Trompeter et al. 2003; Zeitelhofer et al. 2007). Here, we showed that goat GSCs transfected by nucleofection were able to colonize the recipient testes and produce transgenic sperm.

Results

Preparation of donor germ cells for transplantation

Testes were collected from 4 donor goats at the age of 9 weeks and 4 individual germ cell preparations were performed for 4 recipients. After single cell suspensions were prepared by enzyme digestion, 10^9 testicular cells were loaded onto Staptut for germ cell enrichment.

Fractions enriched for germ cells by morphological criteria were pooled and evaluated for cell number, viability, and the percentage of ubiquitin c-terminal hydrolase-L1 (UCH-L1)-positive cells (Table 1). UCH-L1 is a spermatogonia-specific marker that was used to assess the enrichment efficiency and to estimate the percentage of germ cells present in a given cell population (Figure 1). On average, $(1.23 \pm 0.23) \times 10^8$ (n=4) cells were recovered after Staput with a viability of $94.0 \pm 1.6\%$. Of the germ cells, $49.1 \pm 5.7\%$ were positive for UCH-L1, representing a 4- to 5-fold enrichment compared to the crude cell suspension before Staput ($10.7 \pm 1.5\%$ positive for UCH-L1).

Germ cell-enriched cells were then subjected to nucleofection. The transgene construct was chosen because production of human growth hormone in the milk of transgenic animals is of potential commercial interest and therefore best represents a potential application of the technology investigated. The construct (pCBGI-GBC-hGH) used contained the human growth hormone gene (hGH) under the control of the goat beta-casein promoter (GBC). The promoter directs the expression of hGH in the mammary glands for secretion into the milk of transgenic progeny. A chicken beta-globin insulator (CBGI) sequence was engineered upstream of the GBC promoter to insulate the GBC-hGH transgene from any potential position effects upon insertion into the goat genome. The construct was linearized by digestion with SalI and NotI, and vector sequences removed, resulting in an 11-kB linear fragment used for nucleofection. Since goat GSCs cannot be maintained long-term in culture, it was not possible to determine transfection efficiency in vitro.

Due to the limited capacity of nucleofection and the amount of cells required for transplantation, all cells enriched by StaPut ($1-1.49 \times 10^8$ cells per donor, Table 2) were split into 10 nucleofection reactions. For each reaction, 10^7 cells were transfected with 5 μ g of pCBGI-GBC-hGH using Amaxa® Cell Line Nucleofector® Kit V and the program L029. After nucleofection, cells were transferred immediately to a pre-warmed tissue culture dish containing 9 ml of DMEM/12 medium with 5% fetal bovine serum (FBS) (without antibiotics). Cells were allowed to recover at 37°C, 5% CO₂, for 4 hours. Cells from 10 nucleofection reactions were pooled and assessed for cell recovery, cell viability, and the percentage of UCH-L1 positive germ cells (Table 1). Cell recovery was $(33.70 \pm 4.06) \times 10^6$ (n=4) after nucleofection, giving a recovery rate of $32.9 \pm 3.2\%$ from the initial input. The viability of recovered cells at 4 hours post-nucleofection was $84.1 \pm 2.0\%$. As $32.0 \pm 2.9\%$ of recovered cells were UCH-L1 positive, we estimated that $(10.68 \pm 0.45) \times 10^6$ germ cells were recovered after nucleofection and used for transplantation. To allow for an even distribution of cells in the recipient seminiferous tubules upon transplantation, we combined transfected cells with non-transfected cells from other Staput fractions to give to a total of 1.5×10^8 cells in a 3 ml suspension. UCH-L1 staining indicated that there were $21.1 \pm 1.9\%$ germ cells present in the final cell suspension for transplantation. This decrease in germ cell count was due to the mixing of enriched, transfected cells with non-enriched cells. Transplantation is a lengthy process, and thus can negatively impact cell survival; assessment of the cell suspensions at the end of the procedure for cell viability indicated that $87.7\% \pm 4.6\%$ of cells were still viable after transplantation was complete.

Transmission of the transgene to haploid gametes

To evaluate the transmission of the transgene into sperm, semen collections from recipients were started from 9 months after transplantation and lasted for a period of over a year (a total of 62 ejaculates from 4 recipients; Table 2). Ejaculates were evaluated under standard parameters such as volume, sperm number, and progressive motility, and were within expected limits for goat semen. Genomic DNA was extracted from ejaculates and all samples were genotyped by PCR for the presence of the transgene (Figure 2, Summarized in Table 2). Specific PCR primers were designed for the hGH, CBGI, and GBC sequences. The presence of both the hGH and CBGI sequences was used to assess incorporation of the

functional unit (CBGI-GBC-hGH) of the transgene construct in the genome. As the GBC sequence is native to the goat genome, GBC primers were used as a positive control for PCR. Given the fact that colonization efficiency of transfected GSCs in goats are unknown, and transgenic sperm fractions were diluted by non-transgenic sperm produced from non-transfected transplanted germ cells and from recovered endogenous germ cells, nested PCR was employed for hGH and CBGI. Of 62 ejaculates, $63.9 \pm 17.3\%$ were positive for hGH and $42.5 \pm 12.0\%$ were positive for CBGI. In $31.3 \pm 12.6\%$ of ejaculates, both hGH and CBGI were present. We suspect that the discrepancy in the percentage of samples that were positive for hGH or CBGI was due to fragmentation of the transgene construct before or recombination upon genomic integration.

Discussion

GSCs divide very slowly in vitro and are refractory to gene delivery mediated by lipid-based transfection reagents. Although viral vectors such as retroviruses, including lentiviruses, have proven to be effective in delivering transgenes into GSCs (Hamra et al. 2002; Kanatsu-Shinohara et al. 2004; Nagano et al. 2000), they have certain disadvantages, such as limited transgene size, safety concerns, and high cost. This is especially true for the production of biopharmaceutical proteins in the milk of transgenic large animals. In this case the utility of viral transduction is limited, as the potential for residual viral sequences would not meet the stringent requirements for recombinant protein production destined for use in patients.

Nucleofection presents a technical advancement over traditional electroporation technology, with improved transfection efficiency and survival rate. Nucleofection not only allows delivery of large transgene constructs and transfection of non-dividing cells, but also provides a safe, practical alternative to viral vectors for gene delivery (Lorenz et al. 2004; Trompeter et al. 2003; Zeitelhofer et al. 2007). Nucleofection for successful transfection of primary germ cells for transplantation has not been reported in any species. In mice and rats, it has become possible to maintain GSCs in culture for extended periods of time (Kanatsu-Shinohara et al. 2005; Kanatsu-Shinohara et al. 2003; Nagano et al. 2003). Nucleofection has been used successfully to transfect these cell lines prior to transplantation (Izsvak et al. ; Kanatsu-Shinohara et al. 2006). Transfection of established cell lines, however, is less sensitive to adverse effects of electroporation as these cells readily multiply in vitro and cell numbers are not as limited as in primary cells. GSC lines have not yet been established in non-rodent animals, limiting the approach to transfection of primary cells. Non-viral transfection of primary germ cells resulting in transgene transmission after transplantation has not previously been reported. Therefore, the current study is the first report of transgene transmission after nucleofection of primary germ cells followed by transplantation to a donor testis.

In the current study, we demonstrated that primary goat GSCs can be transfected by nucleofection, and that these transfected GSCs were able to colonize recipient testis, establish spermatogenesis, and produce transgenic sperm in recipient goats. We previously demonstrated that transplantation of AAV-transduced germ cells resulted in transgene transmission to embryos when used for in vitro fertilization, indicating that the transgene was not lost through multiple rounds of cell division leading from GSCs to sperm to embryos (Honaramooz et al. 2008). Therefore, while in the current study sperm produced by recipient goats were not used for in vitro fertilization or breeding, it can be assumed that the transgene is stably integrated as it was maintained throughout spermatogenesis and would be transmitted to the embryo at fertilization.

Transgenesis through the male germline stem cells has tremendous potential in species such as goats and pigs, where embryonic stem cell-based transgenic technology is not yet

available and currently practiced methods for producing transgenic animals are generally inefficient. Introduction of genetic modifications in the germline can circumvent problems associated with manipulation of early embryos and developmental abnormalities associated with somatic cell nuclear transfer and reprogramming (Bacci 2007; Niemann et al. 2005). Moreover, spermatogenesis in vivo provides a natural selection scheme to eliminate undesired mutations that disrupt fundamental cellular processes and the mutations that are detrimental to spermatogenesis. Only GSCs are able to colonize the recipient testis and to re-establish spermatogenesis in an infertile recipient testis. When a genetic mutation that is incompatible with survival, proliferation, and differentiation of GSCs is introduced in GSCs, those GSCs will fail to form functional transgenic sperm that carry the lethal mutation. In addition, once transgenic GSCs re-colonize the seminiferous tubules of the recipient testis, they can continuously produce transgenic sperm over the reproductive lifespan of the recipient. Finally, by transplanting transduced GSCs into prepubertal recipients, the time to production of transgenic sperm is shortened compared to production of a transgenic founder animal by somatic cell nuclear transfer.

There are several recent advances that will greatly enhance the utility of germline-mediated transgenesis for the production of large-animal knockouts for modeling human diseases and biomedical research. These include transposon based transduction of GSCs and zinc-finger nuclease technology (Ivics et al. 2011; Izsvak et al. 2010; Watanabe et al. 2010). Transposons have recently been demonstrated to result in transgenesis with high efficiency in rats (Ivics et al. 2011; Izsvak et al. 2010), and the utility of zinc-finger nucleases for genetic modification in a large-animal model has been demonstrated in pigs (Watanabe et al. 2010). In summary, the current study provided proof-of-concept that non-viral transduction of primary goat germ line stem cells by nucleofection prior to transplantation to recipient testes results in transgene transmission to sperm. While overall transmission efficiency was relatively low, future work can address individual factors affecting efficiency, such as use of established GSC lines when available, enhancing donor cell colonization of recipient testes, and improving cell viability after nucleofection. This study therefore provides the foundation for future applications combining non-viral transduction with emerging technologies for targeted genetic modifications in large animals to create animal models for biomedical research or biopharmaceutical production.

Materials & Methods

Preparation of recipient animals

Four recipient goats (J0780, J0783, J0795, J0806) were prepared as described previously (Honaramooz et al. 2005). Briefly, 8 week-old male, Alpine recipient goats received fractionated irradiation of the isolated testes with daily doses of 2 Gy for three consecutive days under short-acting anaesthesia. Germ cell transplantations were performed at 8-9 weeks post-irradiation. We have previously shown that homologous transplantation using unrelated goat donor cells in recipient goat testes did not result in immune rejection of transplanted cells, therefore, recipient goats do not need to be genetically matched to the donor or immune-suppressed (Honaramooz et al. 2003; Honaramooz et al. 2002).

Preparation of donor cells

Donor cells were collected from the testes of 9 week-old dairy goats (n=4). Single cell suspensions were prepared using a sequential enzymatic digestion protocol as described previously (Honaramooz et al. 2002). To enrich spermatogonia (including GSCs), testicular cell suspensions were subjected to Staph velocity sedimentation as previously described (Luo et al. 2006). Fractions were collected and evaluated for enrichment in spermatogonia using a spermatogonia-specific marker, Ubiquitin C-terminal Hydrolase-L1 (UCH-L1) (Luo

et al. 2006). Spermatogonia-enriched fractions were pooled and assessed for cell number and viability by trypan blue exclusion.

Nucleofection

Nucleofection was performed with the Nucleofector II electroporator system (Amaxa, Lonza Inc. Basel, Switzerland). The nucleofection solution and program were chosen based on preliminary assessment of transient transfection efficiency in experiments with pmaxGFPTM (Amaxa), a plasmid containing a GFP reporter gene. For each nucleofection reaction, 10^7 enriched cells were transfected with 5 μ g of the pCBGI-GBC-hGH vector using Amaxa® Cell Line Nucleofector® Kit V (Amaxa) and program L029 (Lonza), according to the manufacturer's protocol. The pCBGI-GBC-hGH vector is an 18.6-kb plasmid containing the human growth hormone gene under the control of the goat beta-casein promoter (GBC) and a chicken beta-globin insulator (CBGI) sequence upstream of the promoter (Figure 3, Supplementary Figure 1). The plasmid was linearized by digestion with SalI and NotI, and vector sequences removed prior to nucleofection. After nucleofection, the transfected cell suspension was transferred to a pre-warmed tissue culture dish containing 9 ml DMEM/F12 medium with 5% fetal bovine serum (without antibiotics). Cells were allowed to recover at 37°C in 5% CO₂ in air for 4 hours, followed by assessment of the cell recovery rate and viability by trypan blue exclusion.

Germ cell transplantation

Cells were collected for germ cell transplantation 4 hours post nucleofection. Cells were washed by centrifugation and resuspension in another 10 ml of medium. For each recipient testis, cells from 10 nucleofection reactions were pooled and mixed with non-transfected cell fractions for a total of 1.5×10^8 cells in 3ml cell suspension. Transplantation was carried out by ultrasound-guided cannulation of the rete testis with delivery of cells by gravity flow, as described previously (Honaramooz et al. 2003; Honaramooz et al. 2008). One testis from each recipient goat (n=4) was injected; the un-injected testis was removed by unilateral castration. Starting nine months after transplantation, goats were trained for semen collection and semen was collected once a week for over a year using an artificial vagina in the presence of a female goat. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania, and performed in accordance with relevant guidelines and regulations. Ejaculates were assessed for volume, sperm concentration and motility by phase contrast microscopy.

Genotyping of semen samples

Ejaculated sperm were washed twice by dilution 1:10 in phosphate-buffered saline (PBS) and centrifugation at 1000 x g. Sperm were processed for DNA extraction, and sperm DNA was analyzed by PCR. Genomic DNA was extracted from sperm using a commercially available kit (QiAmp DNA Mini kit, Qiagen Science, Valencia, CA). For the isolation of sperm DNA, 10^7 sperm/ejaculate were incubated for 1 hour at 37°C in the presence of 100 mM dithiothreitol in lysis buffer prior to DNA extraction. All samples were analyzed in triplicate. Primer sets were designed for the human growth hormone gene (hGH), the goat beta-casein promoter (GBC), and the chicken beta-globin insulator (CBGI). The primer sequences and product sizes are listed in Table 3. Standard PCR was performed for GBC with the following condition: 94°C for 2min, 33 cycles of (94°C for 30 sec, 61.5°C for 30 sec, 72°C for 40 sec), 72 °C for 5 min. For hGH and CBGI, nested PCR was performed. The first round of PCR amplification was done using the standard PCR condition mentioned above and the primer sets F1/R1. One-tenth of the PCR product was used for the second round of amplification using primer sets F2/R2, and the following PCR condition: 94 °C for 2 min, 32 cycles of (94°C for 30 sec, 61.5°C for 30 sec, 72°C for 35 sec), 72 °C for 5min.

Immunofluorescent staining of UCH-L1

A spermatogonia-specific marker, UCH-L1, was used to evaluate the percentage of spermatogonia at each stage of cell preparation. Cells were sampled from the following stages: 1) single cell suspensions before Staput; 2) pooled Staput-enriched fractions; 3) transfected cell preparations at 4 hours post nucleofection; and 4) cell suspensions used for germ cell transplantation. Cells (500,000 cells in 0.5 ml of DMEM per chamber) were allowed to attach to poly-L-lysine-coated Permanox slide chambers for 2 hours, and fixed in 2% paraformaldehyde for 30 minutes. Samples were blocked with 10% normal donkey serum for 2 hours at room temperature and incubated in primary antibody against UCH-L1 (1:1000; Biogenesis, Exeter, NH) overnight at 4°C, followed by Cy3-AffiniPure Donkey anti-Rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as the secondary antibody for 30 minutes at room temperature. Slides were washed and mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescent images were captured on a Leica CTR5000 microscope (Leica Microsystems, Inc., Bannockburn, IL) with a CoolSNAPfx digital camera and Image-pro plus (Media Cybernetics Inc, Bethesda, MD). At least 500 cells per chamber were counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

CBGI	chicken beta-globin insulator
GSCs	germline stem cells
GBC	goat beta-casein promoter
hGH	human growth hormone
SCNT	somatic cell nuclear transfer
UCH-L1	ubiquitin c-terminal hydrolase-L1

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Figure 1. Immunocytochemical detection of UCH-L1 in goat spermatogonia. A) Goat testis tissue. Anti-UCHL-1 and H&E staining. B) Cell suspension after StaPut enrichment. Arrows indicate UCH-L1 positive spermatogonia. Anti-UCHL-1 and DAPI staining. Bars = 50 μ m.

Figure 2.

Genotyping results from representative ejaculates from 4 recipients.

Three representative ejaculate samples from each recipient are shown. Samples were either double-positive for hGH and CBGI, or only positive for hGH. Lanes 1-3 are from donor J0780, Lanes 4-6 from donor J0783, Lanes 7-9 from donor J0795, Lanes 10-12 from donor J0806, Lane 13 is from a control goat. Lane 14 is the positive control using the pCBGI-GBC-hGH vector as the PCR template. Lane 15 is the no-template control, and in lane 16 are the DNA markers. The expected product sizes for hGH, CBGI and GBC are respectively 377 bp, 339 bp and 327 bp.

Figure 3.

The transgene construct.

The human growth hormone (hGH) gene was placed under the control of the goat beta-casein promoter (GBC). A chicken beta-globin insulator (CBGI) sequence was engineered upstream of the GBC promoter. Restriction sites are indicated.

Table1

Enrichment of donor cells by Staput and germ cell recovery after nucleofection

Recipient number	Staput		Nucleofection			
	Cell Number recovered X10 ^{^8}	Cell viability	UCH-L1 positive (%)	*Recovery rate (4hr)	Viability (4hr)	UCH-L1 positive (%)
J0780	1.49	92%	55.1%	33.7%	83.3%	30.8%
J0783	1.35	94%	50.3%	34.9%	86.0%	29.0%
J0795	1.00	94%	49.7%	34.8%	85.5%	32.4%
J0806	1.06	96%	41.4%	28.2%	81.7%	35.7%
mean±SD	1.23±0.23	94%±1.6%	49.1%±5.7%	32.9%±3.2%	84.1%±2%	32.0%±2.9%

* Percentage of cells recovered after nucleofection

Table 2

Summary of genotyping results for ejaculates collected from 4 recipient goats.

Recipients	# of ejaculates collected	# of hGH+ ejaculates (%)	# of CBGI+ ejaculates (%)	# of hGH+,CBGI+ ejaculates (%)
J0780	18	13 (72.2%)	6 (33.3%)	4 (22.2%)
J0783	19	12 (63.2%)	7 (36.8%)	5 (26.3%)
J0795	10	8 (80%)	6 (60%)	5 (50%)
J0806	15	6 (40.0%)	6 (40.0%)	4 (26.7%)
mean±SD		63.9%±17.3%	42.5%±12.0%	31.3%±12.6%

Semen was collected for more than 1 year (number of ejaculates collected for each recipient is listed in column 2).

Table 3

Primer sequences and product sizes for PCR to detect transgene transmission

Gene	forward	reverse	PCR product (bp)
hGH	F1: 5' cttcaagagggcagtgccctccaac 3'	R1: 5' gaaggcatccactcacggattctg 3'	456
	F2: 5' aggcttttgacaacgctatgctc 3'	R:2 5' ctgttgagggtgtcgggaatagac 3'	377
CBGI	F1: 5' gccaatcagtgcatcacggagag 3'	R1: 5' acgattgtatgaacatctacatggc 3'	354
	F2: 5' gtgcatcacggagaggcagatcttg 3'	R2: 5' gtatgaacatctacatggcaattctccag 3'	339
GBC	5' atcacagcatgctttgtctgcc 3'	5' cctgaagagtgaagagagtatagac 3'	327