Embryo Biopsies for Genomic Selection

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Abstract

Embryo genomic selection (preimplantation genetic screening) is increasingly being used to select the best embryos within cattle breeding programs. The procedure starts with the collection of a few cells (biopsy) from each of the embryos before they are individually cryopreserved. The biopsy samples are then genotyped, and the genomic estimated breeding value for each embryo is calculated from prediction equations. These are based on algorithms developed from large reference populations of previously genotyped and phenotyped animals. Based on the genomic estimated breeding value, a decision is made whether to thaw and transfer the embryo or not. Due to the recent availability of low-density bovine single-nucleotide polymorphism (SNP) microarrays, this method is now cost effective. The data in this review describe field results and show that the breeding values calculated from the embryo biopsies are reliable enough for selection. Importantly, the embryo manipulation associated with the procedure only has a very limited negative effect on the resulting pregnancy rate. The method can also be used to prevent the transfer of embryos that are carriers of known recessive lethal genetic defects or other chromosomal aberrations. Therefore it can be concluded that embryo genomic selection can be used in breeding programs to accelerate the rate of genetic gain compared to animal-based genomic selection due to an increased selection intensity among full- and half-sib embryos. Although this review only describes results in dairy cattle, embryo genomic selection can also be used in beef cattle and other livestock species where accurate genomic prediction equations exist.

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5.1 Introduction

Genomic selection is increasingly being used in dairy cattle breeding programs all over the world. Due to very large reference (training) populations in Europe (~40,000), the United States (~60,000), and New Zealand (~6000), reliabilities of the genomic breeding values approach 65–75% for most traits.

Genomic selection is now commonly used to select young animals just after birth or to identify the best bulls and bull mothers to generate future sires for the artificial insemination industry. These genomic assessments and selections are typically conducted on existing, live animals. However, selection based on genomics can also be performed on the early embryo, before transplantation to a recipient female. The advantage of this approach is that a large number of full- and half-sib embryos can be easily produced and only the best embryos of the desired sex and genotype are selected for transfer. In addition, embryos carrying known recessive lethal genetic defects can also be detected and excluded, thereby lowering the number of carrier animals in the population.

The optimal use of genomic selection in an intensive embryo breeding program will accelerate the rate of genetic progress by further increasing selection intensity and reducing generation interval (especially with embryos produced from juvenile animals). The herd improvement cooperative CRV (Arnhem, the Netherlands) has a significant European Holstein Friesian breeding program and produced around 8000 embryos in 2015 (4000 in vivo-flushed embryos and 4000 in vitro-produced [IVP] embryos). Increasingly CRV and other breeding companies are utilizing embryo genotyping to select those embryos possessing the greatest genetic merit for transfer. This is especially in situations where the numbers of recipients are limited, and selecting only the best embryos based on genomics for transfer offers considerable economic advantages.

5.2 Biopsy Methods

In order to perform a DNA test on an embryo, a sample of cells is required. There is a compromise between taking enough cells to enable an accurate DNA test without reducing the developmental competency of the embryo and thus not decreasing its potential to establish a pregnancy. In principle, preimplantation-stage embryos from the two-cell stage onward can be biopsied. However, for practical reasons in cattle, typically only morula- or blastocyst-stage embryos are biopsied for DNA testing. These stages possess a greater number of cells (32–150) and strike a balance, whereby a biopsy of a few cells can be obtained without overly compromising development of the remaining embryo.

There are different methods available for obtaining biopsies from early embryos. Two of the more common methods are (1) the blade biopsy method to cut a portion of a compacted morula or the polar trophectoderm from blastocyst-stage embryos and (2) the needle biopsy method to aspirate cells from cleavage- or morula-stage embryos. Both methods have their advantages and disadvantages

(Mullaart 2002). Some groups (MasterRind, Personal Communication) have good results with needle biopsies, whereas others (e.g., CRV and Midatest, France, Personal Communication) have better results with the blade biopsy method. In general, the success of the biopsy depends on the training and experience of personnel in specific methods. In addition, especially in cattle breeding where sometimes large numbers of embryos have to be biopsied within a limited time, the practicality and labor intensity of the method are important factors. The ultimate choice of method also depends on the stage of the embryo available to be biopsied. The needle biopsy method is typically more suitable for less-advanced embryos (up to morula stage), where cell adhesion is not as strong. In contrast, the blade biopsy method is better suited for more advanced compacted morula- and blastocyst-stage embryos (Mullaart 2002). Also, the quality of the embryo is an important selection criterion for biopsies. At CRV, only grade 1 quality embryos as categorized by the International Embryo Technology Society (IETS) (Robertson and Nelson 1998) are used for biopsy. With IETS grade 2 embryos, it is commonly observed that the remaining embryo deteriorates after taking the biopsy and has lower viability.

As mentioned above, it is important to collect sufficient cells for DNA testing but not so many that compromises embryo competence. Following nuclear staining with DAPI and counting in the fluorescence microscope, the average blade biopsy obtained from blastocyst-stage embryos possessed about 15 cells, but the variation was large (between 8 and 40 cells). In Fig. 5.1, an example of a nine-cell biopsy is illustrated. There does not appear to be any relationship between the number of cells in the biopsy and the embryo stage, probably indicating operator variation.

There is continued debate whether the cells in the biopsy are a representative sample of the entire embryo. Indeed, it has been reported that a large percentage of IVP embryos, and the cells within the trophectoderm in particular, are mixoploid (Viuff et al. 2002). This is especially relevant for blade biopsies that are obtained from the trophectoderm of blastocysts.

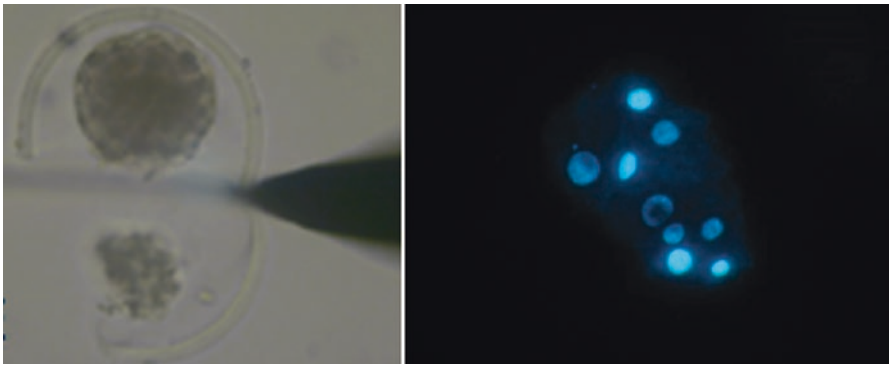

Fig. 5.1 Example of a blade biopsy (left) and DAPI stained biopsy (right) comprising nine trophectodermal cells obtained from a blastocyst

5.3 DNA Amplification

The embryo biopsy can be utilized for various DNA analyses. For instance, it can be used for sex determination, or the identification of specific candidate alleles. But it can also be used for more sophisticated whole-genome analyses, such as genotyping with thousands of single-nucleotide polymorphism (SNP) markers or DNA sequencing. Sex determination by PCR (Bredbacka 1998) is a relatively sensitive assay that only requires a very limited amount of starting material (around ten cells). Blastocyst biopsies therefore provide a sufficient amount of DNA template for the PCR to be immediately performed on the cell sample (and are often done so directly in the field). However, other assays, such as the Illumina SNP chip-based genotyping platform (Illumina, San Diego, CA, USA), require at least 50 ng of DNA. Assuming a cellular DNA content of 4 pg, this corresponds to approximately 12,500 cells. Since the biopsy contains around a thousand-fold less DNA, a "pre-amplification" step is needed before SNP chip-based genotyping can be performed.

This pre-amplification can be done in two general ways: (1) either by culturing the biopsy for several days or (2) by enzymatic pre-amplification. At present, however, only the enzymatic pre-amplification method is used routinely to obtain SNP genotypes from an embryo biopsy. The in vitro culture of embryo biopsies is not yet consistently reliable for cellular amplification in most cases (Ramos-Ibeas et al. 2014; Shojaei Saadi et al. 2014).

For enzymatic pre-amplification, there are various protocols, many of which are based on isothermal multiple displacement amplification (MDA) using Phi29 polymerase. For an overview of different pre-amplification methods, see Shojaei Saadi et al. (2014). It should be noted that the process of taking a small biopsy from an embryo, performing the enzymatic pre-amplification, followed by SNP chip-based genotyping, is a technically challenging process that is error prone. The embryo biopsy (comprising only a few cells) can very occasionally be lost through handling mistakes, but also pre-amplification of the minute amount of template DNA can introduce errors (Ponsart et al. 2013). A major issue with enzymatic pre-amplification is the so-called allele drop-out (ADO) or loss of heterozygosity. This is where only one of the two heterozygous alleles (either paternal or maternal) is pre-amplified and this leads to a false homozygous call at this locus. While ADO tends to be the most common genotyping error, allele drop-ins (gain of heterozygosity) and homozygosity reversal (an erroneous shift from one homozygous genotype to another), although rare, can also occur dependent on the whole-genome amplification method used (Shojaei Saadi et al. 2014).

The whole-genome amplification method used routinely at CRV is the Single-Cell Repli-g Kit (Qiagen, the Netherlands). It has provided the most consistent results, leading to at least a 10,000–20,000-fold amplification of the DNA. This generates sufficient material for further downstream analysis (i.e., SNP chip-based DNA genotyping). However, in a project at AgResearch in New Zealand comparing several different kits, the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, New Zealand) proved to be superior among those tested. The difference between laboratories was most likely due to a subtle differences in

the consistency of the biopsy size (related to sample quality) and experience of the technicians in particular methods.

5.4 DNA Genotyping

After pre-amplification, enough DNA is generated to perform complex DNA analyses, such as on the Illumina genotyping platform with low (7–10K)-, medium (50K)-, or high (777K)-density SNP chips, according to standard protocols. In terms of quality control, the genotyping results are first checked for their call rate. This is a measure for the fraction of the markers on the chip that give a result. So, for instance, a call rate of 0.9 means that genotypes were assigned for 90% of the SNP markers on the chip. As shown in Table 5.1, the call rate and error rate of the genotypes are affected by the number of cells in the biopsy.

The more cells present in the sample at the start of the pre-amplification, the better the genotyping results are. This is most likely caused by fewer errors during the pre-amplification (e.g. less ADO). With more amplification required, a one-cell sample is clearly more sensitive to this than a larger (10–15 cell) sample. With a trophectoderm (blade) biopsy, the average call rate is 0.88 with an error rate of ~1%.

Data from the Fisher et al. (2012) study also indicate that there is a clear inverse correlation between the error rate (measured as the difference between the biopsy compared to the remaining embryo) and the call rate with the 7K density SNP chip (Fig. 5.2).

Table 5.1 Effect of biopsy size on genotype result (modified from Fisher et al. 2012)

Sample	Call rate	Replication error (%)[a]
One-cell biopsy[b]	0.78 ± 0.06	7.8 ± 3.5
Three-cell biopsy[b]	0.86 ± 0.03	2.9 ± 1.7
Trophectoderm biopsy (~10–15 cells)[c]	0.88 ± 0.05	1.1 ± 1.5
Bisected blastocyst[c]	0.94 ± 0.04	0.1 ± 0.1

[a]Based on two or three samples from the same embryo
[b]Based on 50K chip
[c]Based on 7K chip

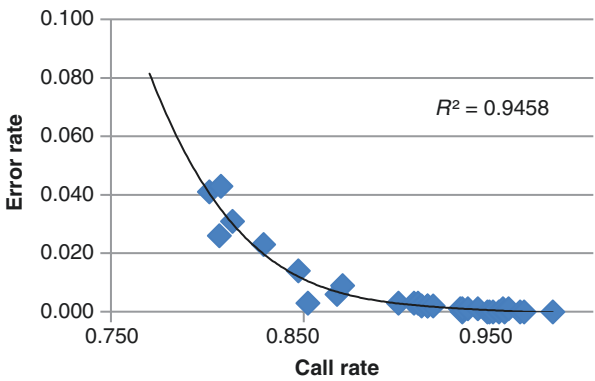

Fig. 5.2 Correlation between call rate vs. error rate

Table 5.2 Mean call rate and percentage of biopsies with a call rate above 0.85 using 10K and 50K SNP chips

Chip type	Number of embryo biopsies	Mean call rate (±SD)	Percentage of biopsies with a call rate >0.85
10K	514	0.84 ± 0.18	80
50K	1378	0.90 ± 0.14	83

From Fig. 5.2 it is also observed that when the call rate is greater than 0.85, the error rate is less than 1%. Comparable results were also obtained using both the 50K and 777K SNP chips (results not shown).

Based on these results, the standard procedure at CRV is to only use genotype results where the call rate is greater than 0.85. A high call rate is a proxy for an inherently lower error rate with SNP genotyping. The effect of call rate on the quality of genotypes and the subsequent calculation of genomic estimated breeding values is also shown in Sect. 5 below.

Over recent years, almost 2000 embryo biopsies have been genotyped by CRV. Initially, the 50K SNP chips were used, but since the cheaper 10K chips became available, they are now used extensively. The results from genotyping these embryos are shown in Table 5.2.

There were no significant differences in the call rates observed between the 10K and 50K SNP chips. As can be seen, the average call rate is between 0.84 and 0.90, and at least 80% of the biopsies gave a call rate above 0.85. Considering that this method is technically challenging (starting with only a few cells), the results are very acceptable and can be used to calculate genomic estimated breeding values for the selection of embryos in commercial breeding programs.

5.5 Breeding Value Estimation

Genotypes from the medium- and high-density SNP chips can be used directly in the calculation of genomic estimated breeding values. However, genotypes from the low-density chip (7–10K) must first be imputed to a reference set consisting of 50K SNPs by using a combination of LinkPHASE, DAGPHASE (for both software packages see Druet and Georges 2010), and Beagle (Browning and Browning 2007). In situations where both parents of the embryo are already genotyped, imputation accuracies are very high (~99%). Genomic evaluation is described by de Roos et al. (2009), where the core of the evaluation is replaced by the method described by Calus et al. (2014). Genomic breeding values are estimated using the EuroGenomics reference database containing more than 35,000 bulls (see also Lund et al. 2011) for 48 different traits including production, health, and fertility, among others.

The genotypes with call rates above 0.85 are used to calculate genomic breeding values, and based on those breeding values, the embryos can be either selected for transfer or discarded. To demonstrate the accuracy of genomic breeding values calculated from embryo biopsies following SNP genotyping, we compared them to the

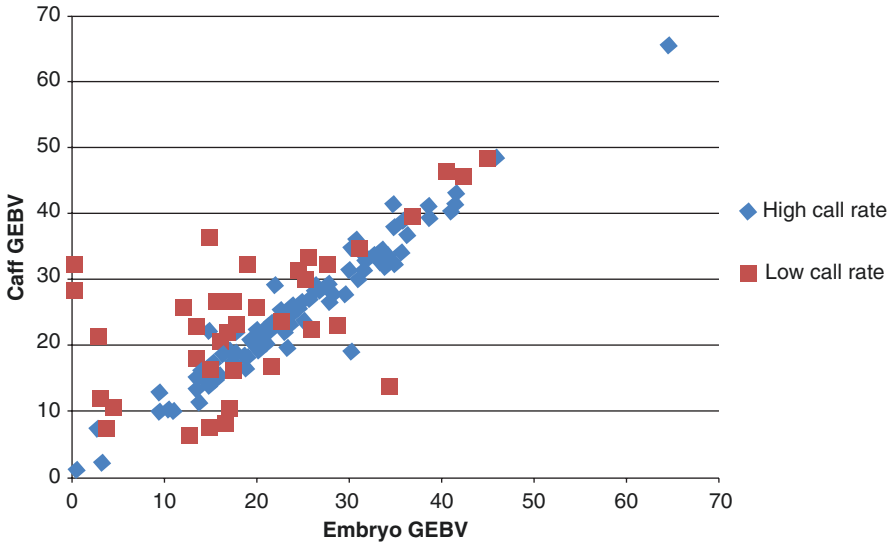

Fig. 5.3 Correlation between the genomic estimated breeding value (GEBV) for kilograms of milk protein (based on 305-day production) in genotyped embryo biopsies with that following genotyping the corresponding calf

corresponding breeding values determined from tissue DNA obtained from each of the resulting calves. In Fig. 5.3, the genomic breeding values for kilograms of milk protein based on genotypes of the embryos is positively correlated with those values obtained by genotyping the corresponding animal after birth. The results show that the correlation is very high ($r^2 = 0.95$) when only genotypes with a call rate above 0.85 are included. If embryo samples with lower call rates are included in the analysis, the correlation is considerably lower ($r^2 = 0.71$). Note that in this case, some embryos with lower call rates were indeed transferred because of their potential value for the breeding program with respect to certain other traits (e.g., polled, red factor, etc.).

The lower correlation for samples with a low call rate was expected, since it was previously shown that lower call rates are associated with a higher error rate and higher ADO (Fig. 5.2). It is also in complete agreement with the results from other groups that demonstrate such correlations to be generally above 0.95 when call rates are high (Ponsart et al. 2013; Shojaei Saadi et al. 2014).

Based on the genomic estimated breeding values, embryos can been ranked in order of superiority and only the highest selected and subsequently transferred. In Fig. 5.4, an example is provided of the genomic breeding values obtained from the different full-sib embryos within a single in vivo flush on Day 7. When no information is available on the genomics of the embryo, all embryos recovered within a single flush have the same "expected" parental average breeding value. However, after genotyping the individual embryos, it became clear that in flush 1, embryo C is predicted to be the most superior for kilogram milk and should be the one selected

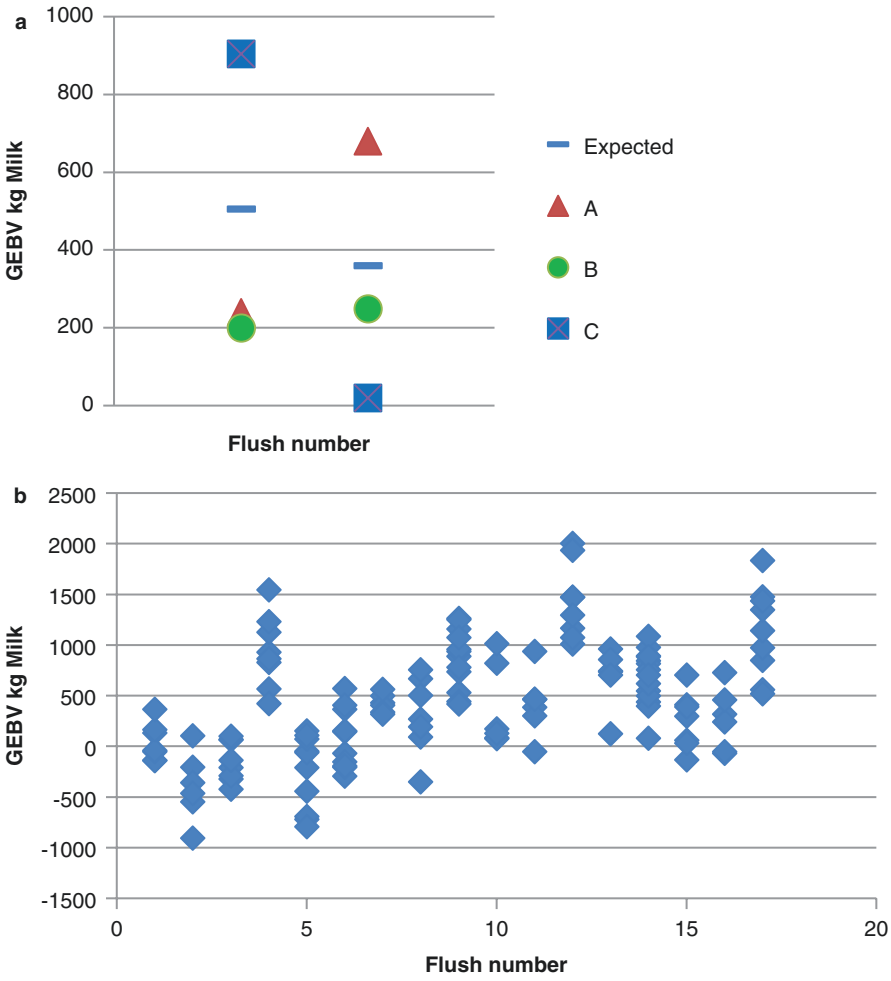

Fig. 5.4 Genomic estimated breeding values (GEBV) for kilogram milk predicted among full-sib in vivo-derived embryos. (**a**) Two flushes, each with three different embryos (A, B, and C), with their expected parental average and GEBV. (**b**) Data from an additional 17 flushes, each with at least six embryos, with their respective estimated GEBV

for transfer (Fig. 5.4a). Likewise in flush 2, embryo A was identified as the best for this particular trait among the three full-sib embryos recovered.

Analysis of additional flushes, with more than six genotyped embryos per flush, showed that there is considerable variation among full-sib embryos (Fig. 5.4b). The average difference within a flush between the embryo with the highest genomic breeding value and the expected breeding value (based on only the parental average) is 446 kg milk (on a yearly production of 8500 kg milk). Within a flush, the average difference between the highest and the lowest is 875 kg milk. This indicates that when all of the embryos cannot be transferred due to limited availability of

recipients or financial constraints, selection of embryos based on genomics can significantly improve genetic progress and reduce embryo transfer costs.

Besides calculating a genomic breeding value, the SNP genotyping data can also be used for other applications. The CRV-customized chip also contains SNPs for sex determination, specific traits, several milk proteins, and certain diseases. Based on X- and Y-chromosome-specific markers, the sex of the embryo can be determined. This sex determination can assist decisions in breeding programs to specifically transfer embryos of the desired gender. For instance, the male embryos can be kept within a company's breeding program and transferred at specific recipient farms, while the females can be sold to the farmer. Also, other simple traits including coat color, polled, and certain milk protein variants (e.g., kappa casein A and B, beta-lactoglobulin A and B, etc.) can be determined for each embryo. This can be important for breeding programs focused on specific traits.

In addition, SNPs for various genetic defects (e.g., BLAD, CVM, lethal haplotypes, etc.) are present on the chip. Based on these, the status with regard to several known genetic diseases for each embryo can be determined. This is very important for two reasons. First, it allows the use of top sires or cows that are otherwise carriers of a certain genetic disease. In such cases, the embryos from these matings can be screened, and only those free of the genetic mutation are transferred. Secondly, it will result in higher pregnancy results, since embryos that are homozygous for certain lethal mutations, and will not survive in vivo, are not transferred.

5.6 Pregnancy Rates of Biopsied Embryos

It is obviously very important to not compromise the embryo by removing too many cells in the biopsy (see Sect. 2). Since the introduction of embryo genotyping at CRV, we have transferred, in excess of 1000 biopsied, in vivo-derived embryos following conventional slow freezing. The freezing of the embryo after biopsy is necessary since the current procedure entailing the pre-amplification of DNA from the biopsy, genotyping, and the calculation of genomic estimated breeding values may take several days. Moreover, in some countries there may be a greater requirement for seasonal calving patterns necessitating cryopreservation of biopsied embryos produced over several months and subsequent transfer of selected embryos to generate spring-born calves. The pregnancy results after single transfer of biopsied frozen embryos, compared to normal intact (non-biopsied) embryos, are shown in Table 5.3.

These results indicate a significant decrease in pregnancy rate after biopsy and freezing. Nevertheless, the 46% pregnancy rate for biopsied frozen in vivo embryos is still very acceptable in the field.

In contrast to in vivo embryos, the pregnancy results following conventional slow freezing of biopsied IVP embryos in ethylene glycol plus sucrose are currently less than ideal. Although our results show that there is negligible impact of slow freezing on the development of intact IVP blastocysts to Day 60 of gestation compared to nonfrozen controls (29/64 = 45% vs. 27/56 = 48%, respectively), the

Table 5.3 Pregnancy results of frozen normal and biopsied in vivo embryos

	Number of embryos transferred	Pregnancy rate[a]
Not biopsied	13,067	54%[b]
Biopsied embryos	1190	46%[b]

[a]Pregnancy as determined by scanning 5 months after transfer
[b]$P < 0.05$

cryosurvival of slow-frozen blastocysts following trophectoderm biopsy is typically much lower (9/54 = 17%; Oback et al. 2017).

The ultrarapid cooling and warming rates afforded by various embryo vitrification methods appear to provide a potential solution. Preliminary results with biopsied vitrified IVP blastocysts indicate embryo survival on Day 65 (43/96 = 45%) to be significantly better than following slow freezing and comparable to fresh control IVP embryos (Fisher et al. 2012; Oback et al. 2017). More experiments are necessary to prove this in practice on a large scale, especially with more user-friendly methods for field situations.

In implementing an embryo genomic selection program, it is critical to optimize each of the manipulation steps to maximize subsequent embryo survival. There is additional expense associated with biopsy and genotyping, and although savings are made by not transferring embryos of lower breeding value, it is important to increase the probability of generating a calf from each selected genotype.

5.7 Optimizing Survival of Genomically Selected Embryos

While much research aims to identify noninvasive biomarkers predictive of oocyte and embryo competence (e.g., metabolomics in spent embryo culture media), a physical sample of the embryo enables direct determination of not only its genotype but also its karyotype, epigenotype, and transcriptome that might all be related to developmental outcomes (Orozco-Lucero and Sirard 2014). In this regard, embryonic cell biopsies are superior and more versatile compared to genomic analyses with fragmented DNA collected from blastocoelic fluid, despite this being a less invasive procedure (Zhang et al. 2016).

Based on the genotype profile consisting of thousands of DNA markers spread evenly throughout the genome, SNP microarray analysis, and ultimately methods utilizing next generation sequencing, enables a form of molecular karyotyping from biopsies. The extent of chromosomal anomalies in the blastomeres of early mammalian embryos is now being revealed by SNP-based karyotyping (Destouni et al. 2016; Treff et al. 2016). However, these SNP-based karyotypes are complicated by any heterogeneity within the sample. This is a particular issue in embryos that are commonly mixoploid (Viuff et al. 2002) and sometimes even chimeric (Garcia-Herreros et al. 2010). It has been reported that on average 11% of trophectoderm cells were polyploid in 96% of Day 7–8 bovine blastocysts (Viuff et al. 2002). While the frequency was less in the inner cell mass, the issue of

mixoploidy raises the question about how representative a small trophectoderm biopsy is of the whole embryo. Despite this concern, the results presented in Sect. 5 show that in practice there is a very high correlation between the breeding values calculated based on a few embryonic cells compared to those from the corresponding calf, especially when imputation is utilized. Furthermore, the developmental consequences of relatively low levels of mixoploidy in blastocysts remain equivocal (King et al. 2006). Nevertheless, chromosome screening avoids the transfer of aneuploid embryos that are unlikely to result in a successful pregnancy (Scott et al. 2013).

The embryo biopsy can also be used to directly determine gene expression profiles that may be predictive of in vivo survival and so assist selection decisions on which embryos to transfer. The possibilities and challenges to identify these molecular markers have been reviewed elsewhere (Bermejo-Alvarez et al. 2011; Orozco-Lucero and Sirard 2014). Studies have identified genes that were either up- or downregulated in biopsies (representing a portion of both the inner cell mass and trophectoderm) that were retrospectively pooled, depending on the subsequent pregnancy outcome (El-Sayed et al. 2006; Ghanem et al. 2011). In the situation where only a trophectoderm biopsy is taken, utilizing informative lineage-specific transcripts prognostic of developmental fate, as well as sharing the precious sample for genomic analyses, remains a considerable challenge for reproductive biotechnologies.

With the present reliance on subjective morphological assessment of embryo quality, the relatively low chance of obtaining a live-born calf from each individual genomically selected embryo is a significant limitation. While there is a negligible decrease in embryo survival as a result of biopsy and vitrification (see Sect. 6), each IVP embryo still only has around a 45% chance of resulting in a viable calf. Future improvements in IVP systems aim to increase the developmental competence of embryos and, combined with identifying competent recipients (McMillan and Donnison 1999), increase pregnancy rates toward some biological limit or to at least result in more consistent outcomes (Vajta et al. 2010). However, even with ideal recipients, some embryos will have an inherently poorer chance of survival due to chromosomal errors or aberrant developmental programming, incompatible with a viable pregnancy. Notwithstanding these cases, options to multiply each genomically selected embryo may increase the chance of obtaining a live-born calf, or calves if required, from that desired genotype. This might include simply bisecting the remaining embryo following biopsy and cryopreservation and then transferring both demi-embryos (Oback et al. 2017). Other methods include utilizing nuclear transfer to multiply elite embryos from donor blastomeres (Misica-Turner et al. 2007) or embryonic cultures derived from either the biopsy (Ramos-Ibeas et al. 2014) or (pluripotent) cultures of the inner cell mass (Verma et al. 2013). An alternative approach may be to transplant embryos and recover the resulting fetuses at a few weeks of age, in order to establish fetal cell lines for subsequent genotyping. Somatic cell nuclear transfer can then be used with selected frozen cell stocks to potentially obtain large numbers of calves of the desired genotype from the original embryo (Kasinathan et al. 2015).

Conclusions

It can be concluded that embryo genotyping from a representative biopsy sample has clear advantages for cattle breeding programs in the following ways:

(a) Predicting the gender of the embryo
(b) Predicting the status for specific phenotypes, for example, coat color, polled, milk protein variants, etc.
(c) Predicting the status for genetic disease
(d) Predicting the genomic breeding value for economically important traits

Predicting genomic breeding values is especially relevant where there is a shortage of recipients and a selection has to be made in the embryos that can be transferred. Furthermore, in cases where embryos are transferred on a recipient farm with a fixed capacity, knowing which embryos are the best genotypes is very important to accelerate the rates of genetic gain achievable and reducing the costs associated with producing elite sires.

The combination of genomic and reproductive technologies provides the option to produce large numbers of low-cost IVP embryos from multifactorial in vitro matings, genotype them, and then only transfer the best. Thus, the current rate of genetic gain can be further accelerated by increasing the selection intensity among multiple full- and half-sib embryos produced from elite parents, compared to the single progeny born from conventional breeding with animal-based genomic selection (Ponsart et al. 2013). Another advantage of embryo genotyping is that it allows for the careful use of bulls that are in fact carriers of a genetic disease. After embryo genotyping, only those embryos free of particular disease-causing or lethal mutations are transferred, essentially recovering otherwise valuable genetics.

Finally, to increase the chance of obtaining a calf (or calves, if required) the selected embryo might be bisected or otherwise multiplied following nuclear transfer of donor blastomeres or embryonic or fetal cell cultures derived from the original embryo. However, the current efficiencies of these procedures still need to be increased for routine commercial use.

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