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Genomic DNA PCR analysis to assess xenograft development in mouse mammary gland

Etienne Aujean¹, Johann Laubier¹, Nicolas Brun¹, Laurence Finot², Eric Chanut², Frédéric Dessauge², Cathy Hue-Beauvais¹ & Fabienne Le Provost^{*1}

ABSTRACT

The mouse transplantation model remains the most relevant methodology to assess the functional capacities of mammary cells and is particularly appropriate for investigations regarding mammary stem cells, whatever the species studied. Following xenotransplantation in mice mammary fat pad, the development of the xenograft is commonly evaluated by immunohistology. Here, we present a simple and rapid method to control the species specificity of a xenograft based on genomic DNA PCR amplification. DNA is extracted from the fixed samples intended for histology, thus allowing the reuse of precious samples. Standard and digital droplet PCR (requiring low DNA quantities) methods have been used to make the present method suitable for the analysis of xenotransplanted samples.

METHOD SUMMARY

A new approach to estimate the species specificity of xenografts is described. This method is based on PCR amplification of genomic DNA samples extracted from mammary tissue that has been processed for histological analyses, such as whole mounts, paraffin blocks and sections.

KEYWORDS

bovine • genomic DNA extraction • histology • mammary gland • murine • species-specific PCR amplification • xenotransplantation

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When studying epithelial cell lineages and stem cells of the mammary gland, the mammary transplantation approach is recognized as the most relevant technology for testing the capacities of the isolated cells or explants to reconstitute a functional organ [1,2]. This method consists of transplantation of mammary explants or sorted cells in cleared mouse mammary fat pad [3] and is widely used to characterize mouse mammary epithelial cells, particularly the stem and progenitor cell populations. More recently, xenotransplantation experiments have been extended to studies involving mammary stem cells from species such as humans and cattle [4–6]. However, in xenotransplantation assays, it is critically important to confirm that the developing tissue belongs to the species of origin. Kuperwasser *et al.* [4] performed this species-specificity control through immunohistochemistry with specific anti-human antibodies, as well as genomic FISH analyses. Unfortunately, specific antibodies to bovine proteins are scarce; therefore, mouse antibodies have been used in some cases to characterize bovine xenotransplants [6,7]. The lack of specific immunological tools for species such as bovine can make the results of xenograft development difficult to interpret. The PCR method could be used to detect transplanted cells using DNA extracted from frozen tissue [8,9]. Here, we propose an alternative method to determine the species-specific mammary tissue that develops after xenotransplantation using genomic DNA extracted from fixed tissue. This method can be applied regardless of the species of the graft of origin and independently of the immunological analysis.

The method was developed to characterize the ability of the bovine-transplanted samples to generate mammary epithelium. Mouse mammary glands were collected 8 weeks after transplantation, and whole mounts were carried out. Briefly, the mammary tissues were excised from eutha-

nized mice, spread on glass slides, fixed in Carnoy's solution (ethanol, chloroform and glacial acetic acid in a 6:3:1 ratio) overnight at room temperature, and rehydrated gradually. The tissues were stained with Carmin alum solution for 1.5 h at 4°C, dehydrated, cleared in xylene or ESTISOL™ (Estichem A/S, Gadstrup, Denmark) solution and mounted using Permount™ (Thermo Fisher Scientific, MA, USA) mounting medium. After image acquisitions, tissues present in the whole mounts were either used for DNA extraction or embedded in paraffin blocks, sectioned (5-µm thick sections) and stained with hematoxylin and eosin solution.

To extract the genomic DNA from tissues present in whole mounts, samples were dismantled in xylene, then rehydrated and dried at room temperature. To extract the genomic DNA from paraffin blocks or sections, the part containing the tissue was cut manually with a scalpel. The paraffin was then removed in xylene, and collected tissue pieces were washed in 100% ethanol and dried at room temperature. For all samples, tissue was incubated in 500 µl (small pieces, ≤1 cm²) to 1 ml (entire gland) of lysis buffer (100-mM Tris, 5-mM EDTA, 0.2% SDS, 0.2-M NaCl; pH 7.4) supplemented with 1% proteinase K (20 mg/ml) at 37°C for 3 h to overnight according to the tissue size. After centrifugation (10 min at 13,000×g), the supernatant was collected, supplemented with 1 volume of isopropanol and centrifuged as above. The resulting pellet was dried and suspended in 50 µl of 10-mM Tris and 0.1-mM EDTA buffer. DNA quality and quantity were assessed by loading an aliquot in 0.8% agarose gel and by the absorbance determination at 230, 260 and 280 nm using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific).

As the clearing process may impact genomic DNA quality, we compared two clearing protocols using xylene (X) or ESTISOL (E) solutions. For this test, DNA extractions were performed using mammary gland whole mounts from control mice (Figure 1A) and the phenol-chloroform

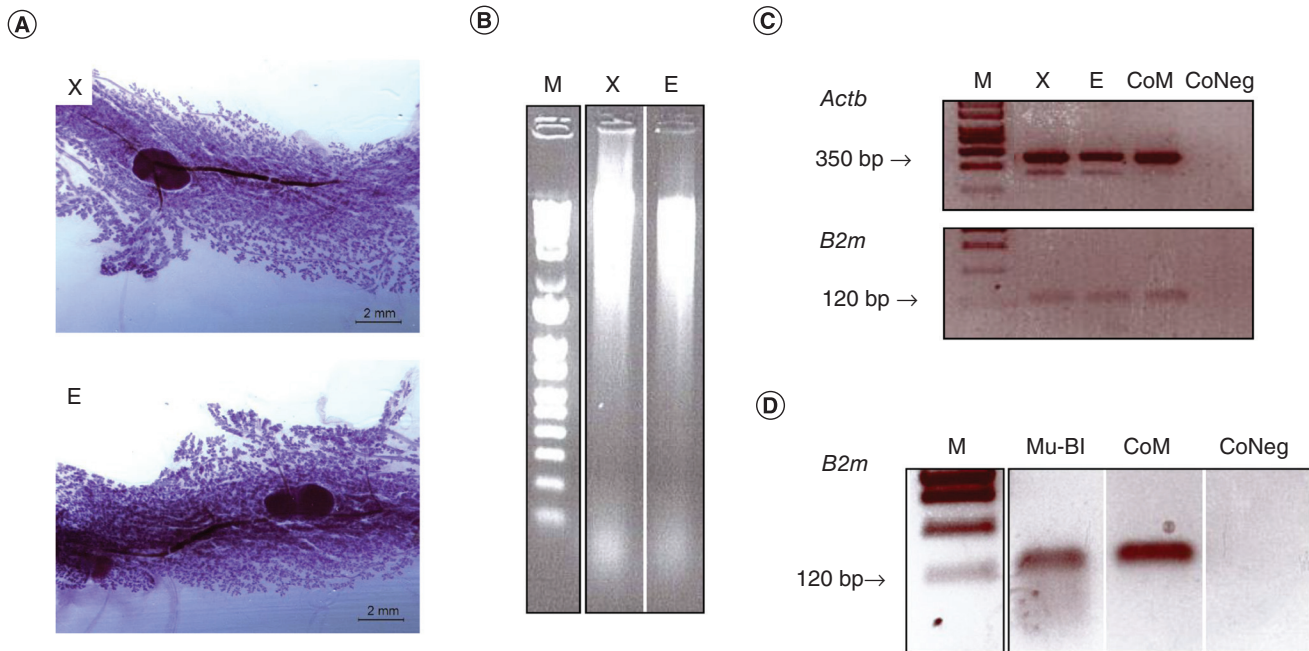


Figure 1. Analysis of the genomic DNA extracted from mouse mammary gland transplanted with mouse mammary explants. (A) Whole mount cleared by X or E solutions. (B) Electrophoretic assessment of genomic DNA (1- μ g loads) extracted from whole mounts cleared by either X or E solutions. (C) Amplification of the murine *Actb* and *B2m* genes from genomic DNA extracted from whole mounts cleared by either X or E solutions. (D) Amplification of murine *B2m* gene from genomic DNA extracted from paraffin-embedded tissue (Mu-BI).

CoM: Control using mouse genomic DNA extracted from tail; CoNeg: Negative control without genomic DNA; E: ESTISOL; M: Promega 1-Kb ladder size marker in part (B); M: Promega bench-top 100-bp (Promega) size marker in parts (C) and (D); X: Xylene.

extraction protocol. In both cases, the quality (Figure 1B) and quantity (620 μ g/sample) of DNA were equivalent; therefore, xylene clearing was further used because it was the most efficient. Mouse *Actb* and *B2m* gene primers (Table 1) allowed the amplification of PCR products of 350 and 120 bp, respectively, from DNA (100 ng) extracted from mouse mammary gland whole mounts by standard PCR (45 cycles) (Figure 1C). In contrast, genomic DNA extracted from paraffin-embedded tissues or sections (3–5 sections per sample) was degraded (Supplementary Figure 1) but did allow amplification of fragments of small size, such as 120 bp (using the *B2m* primers) (Figure 1D), but not amplification of fragments 350 bp in length (using the *Actb* primers; data not shown).

In order to detect the presence of exogenous DNA in xenotransplanted mice, DNA extraction was performed using mouse mammary transplanted with bovine samples (Figure 2A and Supplementary Figure 1). PCR amplifications of species-specific genes were performed on genomic DNA extracted from tissues embedded in paraffin: murine *B2m* gene and bovine *Mtx2* and *Twf1* genes (Table 1). DNA amplifications

were carried out using primers chosen to amplify small fragments (120 bp). The presence of bovine epithelial development was confirmed by classical PCR and digital droplet PCR (ddPCR) methods (Figure 2 & Supplementary Figure 1). Because the parts of the mammary tissue containing the graft were cut manually with a scalpel under a binocular, they contained some mouse tissue. This explains the amplifications obtained using mouse *B2m* gene primers (Figure 2 & Supplementary Figure 1).

Although xenotransplantation is a very attractive tool, the rate of xenograft development is low; hence, each sample is precious. In this context, the strategy reported here offers several advantages. Indeed, the graft development can be characterized by histology (whole-mount and hematoxylin and eosin staining of sections), and its species specificity can be assessed using the same sample. As graft development is sparse and limited, the possibility to select the part of the gland that contains the graft, after histological observation, allows further improvement of the DNA analysis by increasing the concentration of genomic DNA contained in the graft. Here, we propose an alternative approach to

immunohistological analysis: amplification performed on DNA extracted from various types of samples, such as whole mounts or tissue sections, and the use of standard PCR technology, as well as ddPCR, which requires lower DNA quantities.

PCR analysis of genomic DNA is faster, less expensive and more convenient than immunohistological methods. It is also more accurate, as species-specific antibodies are scarce for livestock species. Most of the antibodies currently available are developed against human or mouse proteins and do not cross-react with livestock species. In contrast, the design of species-specific primers is simple, and they can be developed for all species. Therefore, the approach presented here promises to remove important biotechnological bottlenecks.

So far xenotransplantation assays using human or bovine samples have been described in very few studies; however, the number of species under study is expected to increase in the future. Structural and functional characteristics of the mammary gland are conserved across species, but interspecies specificities still remain that are important to highlight. The establishment

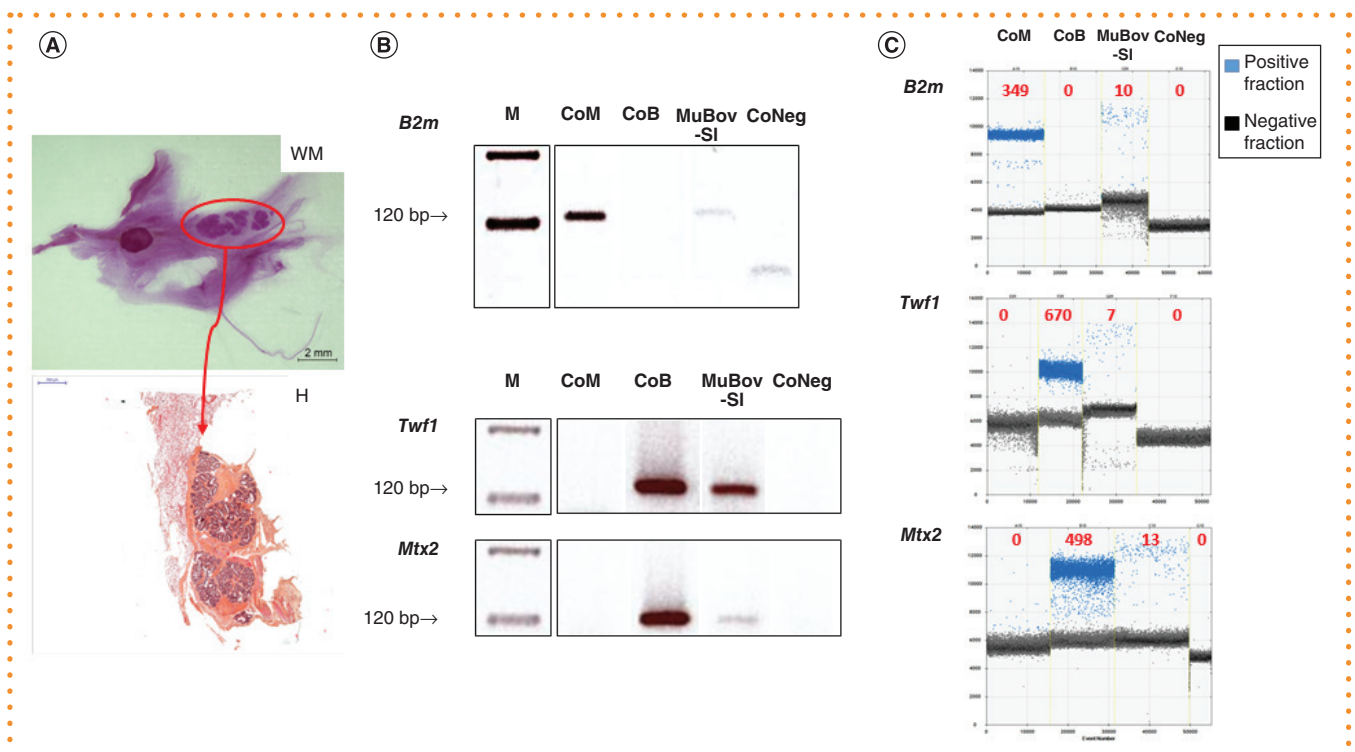


Figure 2. Analyses of genomic DNA extracted from mouse mammary gland xenotransplanted with bovine explants. (A) WM and hematoxylin and eosin staining of H. Red circle shows bovine xenograft. (B) Amplification using classical PCR method of mouse *B2m* and bovine *Twf1* and *Mtx2* genes from genomic DNA extracted from sections MuBov-SI, CoM and CoB. (C) Amplification using ddPCR method of mouse *B2m* and bovine *Twf1* and *Mtx2* genes from genomic DNA (10 ng) extracted from sections, mouse tail and bovine blood. Results are presented as gene copy number per microliter of DNA sample. Red numbers indicate the concentration for each sample (copy number per microliter). CoB: Bovine blood; CoM: Mouse tail; CoNeg: Negative control without genomic DNA; ddPCR: Digital droplet PCR; H: Histological section; M: Promega bench-top 100-bp size marker; MuBov-SI: Mouse mammary gland xenotransplanted with bovine explant; WM: Whole-mount.

of these particular features involves detailed processes of tissue development, including mammary stem cell differentiation. In conclusion, this method represents a simple and reliable way to control the species of origin of the developed tissue during xenotransplantation assays.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0125

AUTHOR CONTRIBUTIONS

E Aujean, J Laubier, N Brun, L Finot and C Hue-Beauvais performed the experiments and analyzed the data. F Le Provost, E Chanat and F Dessauge designed the experiments and wrote the paper.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

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Table 1. Sequences of primers used to perform amplifications by classical PCR and digital droplet PCR methods.			
Species	Gene	Forward primer (5' to 3' end)	Reverse primer (5' to 3' end)
Murine	<i>Actb</i>	GCTGTATCCCCTCCATCGTG	CACGGTTGGCCTTAGGGTTCAG
Murine	<i>B2m</i>	GTGACGACCTCCGGATCTGA	GCCGAGTAGCAGCCACTGAA
Bovine	<i>Mtx2</i>	AGAGTCTTGTACCCCATCCA	ACCCTTGGCCTCTGAATCAT
Bovine	<i>Twf1</i>	CTCGCGATCACTTAACGA	GTGGTGCAAAGACTTACGAACA

ddPCR: Digital droplet PCR.

manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

ETHICAL CONDUCT OF RESEARCH

All animal studies were performed in compliance with European Council Directive 86/609/EEC regulations regarding animal experimentation and with the authorization of the French Ministry of Agriculture (#14945-2018050415489992v3).

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