Title: Robust generation of transgenic mice by hypotonic shock mediated transgene delivery in testicular germ cells

Short Title: A shortcut method of transgenesis

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Abstract:

Although our ability to decipher gene sequences has increased enormously with the advent of modern sequencing tools, the efficiency of obtaining information about functions of new genes have not increased correspondingly. This is mainly due to lack of advancements in methods of transgenesis. Presently practiced zygotic pronuclear DNA-microinjection is time consuming, tedious and requires highly skilled persons for embryo-manipulation. Hence, we have established an innovative technique for making transgenic mice by hypotonic-shock driven gene delivery in male germ cells. For this, suspension of transgene in hypotonic Tris-HCl solution was delivered in testis non-surgically by simple injection. This resulted in internalization and integration of the transgene in dividing germ-cells by hypotonic-shock leading to generation of transgenic progeny by such males. We have also achieved gene silencing by using construct containing shRNA for specific mRNA. Several transgenic animals can be generated within short span of time by this easily adaptable novel technique.

Method Summary:

We present here a novel method for generating transgenic mice by non-surgical in vivo transfection of testis. Transfection is achieved by simple injection of testis with transgene suspended in hypotonic solution of Tris-HCl. We show here transgene is stably transferred in progenies generated from transfected male mice. By this new method we have successfully generated both over-expression and knock-down mice models. This novel technique of making transgenic animal is simple, fast, cost effective and minimally invasive.

Introduction:

Transgenesis is an indispensable technology in biomedical research as it allows to manipulate the genome of an organism at will providing a platform for determining functions of various genes. With the advent of high throughput sequencing technologies, valuable information about several genes which are differentially expressed in various cells and tissues have been gathered. This has provided a huge database of information about the transcriptional programs that control development and maintenance of an organism(1, 2, 3). However, without studies of functional genomics, functions of various differentially expressed genes cannot be known. Although several methods of germ line gene transfer are available to make transgenic animals for this purpose, they are tedious, time consuming and expensive. For instance, classical method of gene transfer involves microinjection of nucleic acids into fertilized eggs, with limited success rate of about 10-40%. Moreover, it is beyond the reach of several investigators, largely due to technical complexities in adopting skills for embryo manipulation (4). This has caused a remarkable delay in functional interpretation of several newly found genes in tissue and age specific manner, limiting the pace of biological research. Since this technique requires several females for super ovulation to obtain large number of fertilized oocytes for such DNA microinjection, it is rarely practiced for transgenesis in large animals (5). Hence, it is imperative to establish alternative, easily adaptable, less invasive and less time consuming technique of making transgenic animals. This will not only help accelerating the studies of functional genomics using transgenic mice but will also increase scope for generating transgenic farmed animals (6).

To this end, we have reported an alternative procedure of electroporation based male germ cell mediated *in-vivo* transgenesis (7). In spite of having superiority over earlier methods of gene transfer, the usage of this technique remained limited. We reasoned that for unskilled person,

performing surgery around reproductive organs and delivery of electric pulses to surgically exposed testis might be challenging. In addition, such procedures involving electric current may not be suitable for transgenesis in large animals (other than rodents). Motivated by the above challenges and to circumvent the caveats resident in previous procedure, here we report the development of an easy and simple procedure for *in vivo* gene transfer in male germ cells of the testis for producing transgenic mice. Delivery of the transgene into germ cells was achieved, by mild hypotonic shock to spermatogonia upon testicular injection of linearized gene suspended in the hypotonic solution of Tris-HCl. It was reported previously that in primary cultures of Sertoli cells contaminated with germ cells, 20 mM Tris-HCl solution rendered hypotonic shock to male germ cells (*in vitro*) causing their rupture and death but spared adjacent Sertoli cells (8). We exploited this property of Tris-HCl and hypothesized that presence of Tris-HCl at certain concentration in testis might provide a hypotonic environment transiently to the germ cells *in vivo*. This would not kill germ cells but allow internalization of surrounding solution or suspension through temporary opening of the pores of their cell membranes. A transgene suspended in such hypotonic solution may get internalized and integrate in native genome of a dividing germ cells. The hypotonicity generated by injected Tris-HCl solution may not last longer within testis as testicular interstitial fluid constantly moves out (9). Hypotonic solution would get cleared from the interstium of testis because turnover rate of interstitial fluid in testis is considerably high (10), thereby causing a temporary shock for a short duration. This easy procedure of *in-vivo* transgenesis provides scope to biomedical researchers for generating their own transgenic animal models.

Materials and Methods

Animals:

FVB/J strain of mice was used for the present study. The mice were housed in a climate controlled environment under standard light (14 hour light, 10 hour dark cycle), temperature (23 ± 1 °C), and humidity (50 ± 5%). Animals were used as per the National Guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA). Protocols for the experiments were approved by the Institutional Animal Ethics Committee.

Plasmids:

pCX-Egfp plasmid was a kind gift from Dr. Y. Junming (University of Tennesse, Memphis, USA), it contains chicken beta actin promoter along with cytomegalovirus transcription enhancer element (CX) and an *egfp* gene. The plasmid was digested with *Sal I* restriction enzyme to obtain a single fragment of 5.5kb which was used for the testicular injection during standardization of the procedure**(SupplementaryFig. 1a)**.

Amh-IRES2-Egfp: For this construct,632bp upstream region of the anti mullerian hormone (Amh) gene spanning from -1bp to -632bp from transcription start site of mouse Amh gene was PCR amplified from mouse genome and cloned in *pIRES2-Egfp* vector to generate *Amh-IRES2-Egfp* construct. *Amh-IRES2-Egfp* plasmid DNA was digested with *Ase*I and *Nhe*I restriction enzymes, the fragment of interest (5.3kb) had Amh promoter at 5' end and *egfp* gene towards the 3' end **(SupplementaryFig. 1b)**.

Bucsn2-IRES2-Egfp*:* This construct expresses *egfp* gene under Buffalo beta casein promoter(11). *Bucsn2-IRES2-Egfp* plasmid DNA was digested with *Pst*I *and Sfo*I restriction enzymes. The fragment of interest (~6.8kb) had BuCSN2 promoter at 5' end and *egfp* gene towards the 3' end **(SupplementaryFig. 1c)**.

***Fetuin-A* –shRNA construct:** *Fetuin-A* shRNA bacterial clones targeting mouse *Fetuin-A* gene were procured from Sigma-Aldrich, USA. The shRNA sequence was cloned in pLKO.1-puro vector (12) between U6 promoter(RNA polymerase III promoter) and central polypurine tract (cPPT). *Fetuin-A* shRNA plasmid DNA was linearized with *Nco*I restriction enzyme to obtain 7.1 kb fragment, which was used to make transgenic mice **(SupplementaryFig. 1d)**.

Hypotonic solution mediated *in vivo* gene transfer in testicular germ cells

Male mice (30±2 days post birth) were anesthetized using intra-peritonial injection (~120 µl) of a mixture of ketamine (45mg/kg) and xylazine (8mg/kg). Hair from scrotal area were trimmed, followed by disinfection with Betadiene. The area was rinsed after 2 minutes with 70% alcohol, leaving the area clean and moist. Gently, testis were pushed down from abdominal cavity with the help of thumb and index finger. Plasmid DNA suspended in Tris-HCl solution (concentrations ranging from 20mM to 200mM during standardization) of pH 7.0, along with 0.04% Trypan blue and was injected slowly in to the descended testis using 26 guage10µl volume Hamilton syringe (701N; Hamilton Bonaduz AG, Switzerland). For standardization, a range of 20-30µl of hypotonic solution containing various concentration of linearized plasmid DNA (0.5-1.5µg/µl) was delivered into single testis. Variation in the concentration of Tris-HCl

solution, concentration of plasmid DNA and number of injection sites (1-4) were done at the time of standardization of this technique.

Generation and screening of transgenic lines

Three constructs for over-expression and one construct for down regulation by shRNA were used for development and validation of this new procedure. The injected mice (fore-founders) were put for natural mating with wild type females after 30-35 days post-injection (mice age 60-65 days) and genomic DNA of progeny were analyzed for the presence of transgene. For this purpose, tail biopsies were obtained at 21 days of age and genomic DNA of mice was extracted from respective tissue. Presence of transgene in the genomic DNA was determined by PCR using transgene-specific primers (**SupplementaryTable. 1**).Genomic DNA from PCR positive pups were further analyzed for gene integration by Slot blot analysis. In brief, probe identifying the transgene fragment was generated by αP^{32}dCTP using High Prime DNA labeling kit (Roche Diagnostic GmbH, Mannheim, Germany). Denatured gDNA (1 µg) was blotted on Hybond N^{+} (Amersham Pharmacia Biotech, England) membrane with slot blot apparatus (Cleaver Scientific, Warwickshire, UK) under vacuum. The membranes were pre-hybridized for 4hours, followed by hybridization with respective transgene specific probes for 10-12 hours. The hybridized blot was exposed to Kodak BioMax MR Film (Kodak, Rochestar, New York, USA) for detection of signals by autoradiography.

161 **Southern blot analysis**

162 Southern blot analysis was performed following standard procedure (13). Ten micrograms of
163 genomic DNA obtained from transgenic progeny of G1 generation was digested with BamHI
164 restriction endonuclease. Digested product was resolved on 1% agarose gel, and transferred to
165 Hybond N+ (GE Healthcare, England). An *egfp* probe fragment of ~600 bp was labelled with
166 αP^{32}dCTP using High Prime DNA labeling kit (Roche Diagnostic GmbH, Mannheim, Germany)
167 and was used to detect the transgene integrations. The membrane was hybridized for 20 hours
168 and was exposed to Kodak BioMax MS film (Kodak, Rochestar, New York, USA) for detection
169 of signals by autoradiography.

170

171 **Histology and Immuno-histochemistry**

172 For histology, tissues were dissected, fixed in formalin and processed for paraffin embedding.
173 Sections were stained by hematoxylin and eosin or with Vonkossa stain. We determined the
174 expression of EGFP in the seminiferous tubule of *pCX-Egfp* and *Amh-IRES2-Egfp* transgenic
175 mice by immuno-histochemistry. Testis sections of 5µm were subjected to immuno staining with
176 mouse anti-GFP (Clontech, Mountain View, CA, USA) as primary antibody at a dilution of
177 1:200 and then anti mouse IgG tagged to alexa fluor 488 (Molecular probes, Eugene, OR, USA)
178 was used at a dilution of 1:250, as secondary antibody. The fluorescence was detected by Nikon
179 Eclipse Ti inverted fluorescence microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan).
180 The images were captured using Nikon-digital sight DS-Ri1 camera.

181

182 **Western blot analysis**

183 Tissue lysates from testis of *Amh-IRES2-Egfp* transgenic mice; mammary gland, liver, spleen,
184 brain, heart and kidney ofBucsn2-IRES2-Egfp transgenic mice were used to determine presence
185 of EGFP. 30µg of protein sample was resolved on 12% SDS-PAGE and transferred to PVDF
186 membranes (MDI, India). The membranes were first incubated with primary antibody (mouse
187 anti-GFP) at 1:1000 dilutions followed by anti-mouse secondary antibody conjugated with
188 horseradish peroxidase at 1:5000 dilutions. The protein bands were detected using enhanced
189 chemiluminescence method (ECL, Amersham Biosciences, UK).

190

191 **RNA isolation and Real-Time PCR**

192 RNA was isolated from liver tissue of *Fetuin-A* knockdown transgenic mice and wild type mice
193 using TRIzol (Sigma Chemical Co., USA). Real time PCR was performed using primer specific
194 for Fetuin-A gene [**5'**TCACAGATCCAGCCAAATGC**3'** as forward primer and
195 **5'**GGAATAACTTGCAGGCCACT**3'** as reverse Primer]. RNA (1µg) was treated with DNase I
196 (1 unit; Fermentas, Pittsburgh, PA, USA) for 30 minutes at 37°C. Reaction was terminated by
197 adding 1µl of 25 mM EDTA and incubating at 65°C for 10 minutes. DNaseI treated RNA was
198 reverse transcribed using Reverse Transcription System (Eurogentec, Seraing, Belgium) with
199 MuMLV reverse transcriptase enzyme and oligo-dT (15mer) for the single-strand cDNA
200 synthesis. Real time PCR amplifications were performed in the Realplex (Eppendorf, Hamburg,
201 Germany) in a total volume of 10µl, which included 1µl of cDNA, 5µl of Power SYBR Green
202 Master Mix (Applied Biosystems, CA, USA)and 0.5µl of each primer. Expression of GAPDH

[using **5'**AGAACATCATCCCTGCATCC **3'** as forward primer and **5'**CACATTGGGGGTAGGAACAC**3'** as reverse Primer] was analyzed for use as an endogenous housekeeping gene control. Relative fold change of Fetuin-A mRNA in transgenic animal with respect to wild type mice was calculated by $2^{\Delta\Delta ct}$ method(14).

Results and Discussion:

For standardization of the procedure, linearized *pCX-Egfp construct* was suspended in Tris-HCl solution and injected into testis to achieve transfection of germ cells, through hypotonic shock. Injection parameters such as amount of plasmid DNA (ranging from 10 µg -30 µg/testis), volume of injection (ranging from 20 µl - 30 µl), number of injections (1-4) in the testis and the concentration of Tris-HCl (ranging from 20 mM to 200 mM) were tested to determine the most suitable condition for achieving a successful *in vivo* germ-line transfection (**SupplementaryTable. 2**). During standardization, we injected the construct *pCX-Egfp*, in which EGFP is expressed under control of chicken beta actin promoter, in one testis keeping contralateral testis as control. We observed that injection at 2 diagnolly opposite sites, of 12.5 µg DNA suspended in 25 µl of 150 mM Tris-HCl in each testis of 30±2 days old FVB male mice yielded best transfection as adjudged by EGFP expression (**SupplementaryTable. 2**). EGFP fluorescence was evaluated in injected testis after 30 days of injection. The EGFP expression was found in several of the germ cells (**Fig. 1A & B**) and there was no discernible damage to testis (**Fig. 1C**). Upon standardization, we routinely injected the transgene in both testes. This was done in order to increase the likelihood of obtaining more transgenic sperm. Breeding of such mice (fore founder) with wild type females, 30 days after gene transfection successfully

generated transgenic progeny. We observed presence of several PCR positive animals in generation one (G1) and generation two (G2) progeny upon genotyping (**SupplementaryFig. 2**). Further, Slot blot analysis also proved presence of transgene in several progeny (**SupplementaryFig. 3**). Once we assured the transgene integration and its transmission to progeny using *pCX-Egfp* construct, we attempted to generate transgenic lines by this method using constructs that was designed to exert tissue and age specific gene expression.

We generated anti mullerian hormone (*Amh*) promoter driven *egfp* (*Amh-IRES2-Egfp)* and buffalo beta casein (*Bucsn2*) promoter driven e*gfp* (*Bucsn2-IRES2-Egfp)* transgenic mice by this procedure. We anlyzed and confirmed presence of *Amh-IRES2-Egfp* by slot blot analysis of PCR-positive animals from G1and G2 generations (**SupplementaryFig. 4a and 4b**). Southern blot analysis were performed to determine transgene integration sites and it was found that in this transgenic mice line, there were mostly two types of integration of the delivered transgene in the genome (**Fig. 2A**). One is integration in tandem repeats which generated ~5.3kb band in southern blot and another is single copy integration for which a band at ~4kb was observed in the same (**Fig. 2A and SupplementaryFig. 4c**).

Amh is expressed at very high levels in immature Sertoli cells of the testis from 12.5 day post coitum (dpc) in the mouse (15) until the onset of puberty at around 10 days of postnatal age (16)**.** We observed Sertoli cell specific expression of EGFP in testis of 5days old transgenic mice (**Fig. 2B)** and absence of EGFP expression in other organs **(SupplementaryFig. 5a).** We also noticed that EGFP expression was lacking at 42 days (post-pubertal) of age (**SupplementaryFig. 5b**). Western blot analysis with protein isolated from testicular tissue of 5 days old transgenic mice of G1 and G2 progeny revealed presence of 28 KDa band corresponding to EGFP (**Fig. 2C**).

In *Bucsn2-IRES2-Egfp* transgenic line, we used *Bucsn2-IRES2-Egfp* construct where *Egfp* was cloned under the regulation of buffalo β-casein promoter (11). Expression of β-casein in mammary gland of the mammals is well reported in the literature previously (Bühler et. al., 1990; Rijnkels, 2002). The fore-founders carrying *Bucsn2-IRES2-Egfp* fragment successfully generated transgenic animals in G1 generation. We evaluated the transgene integration by PCR and slot blot analysis and found many litters positive for the transgene **(SupplementaryFig. 6 and SupplementaryFig. 7a)**. To check the expression of EGFP in the mammary gland, lactating transgenic females were used. This was done to ensure that milk was present in the mammary gland because beta casein promoter is activated upon stimulation by lactogenic hormone after parturition (19). We observed that female mice carrying *Bucsn2-IRES2-Egfp* transgene displayed very intense native GFP fluorescence (observed under fluorescence stereo-microscope) in their mammary glands during period of lactation **(Fig. 3A).** Western blot analysis showed presence of EGFP only in mammary tissue extract and not in other tissue types of the same transgenic female or in the mammary gland of transgenic females generated using other genes, by this procedure **(Fig. 3B** and **SupplementaryFig. 7b)**. These observations are also similar to results described previously (11).

Once the overexpression of transgene was successfully achieved by this procedure, we evaluated the utility of this method for effective knock-down of genes *in vivo* by overexpressing shRNA specific for a gene. For this purpose, we attempted to knockdown Fetuin-A mRNA. Fetuin-A is made in liver and secreted in bloodstream(20)**.** Fore-founder mice in which construct encoding Fetuin-*A* shRNA was successfully transfected into testicular germ cells, produced transgenic progeny, as detected by slot blot analysis (**SupplementaryFig. 8**). Quantitative Real time PCR revealed that Fetuin-A mRNA was drastically reduced in liver of such mice as compared to wild

type mice (**Fig. 4A**). Fetuin-A is a major inhibitor of calcification in soft tissues especially of heart, kidney and lung; deficiency of Fetuin-A is known to be associated with dystrophic calcification of these tissues (21). Upon Von kossa staining of liver and heart tissue obtained from knock-down mice, we observed increased calcification of tissues, unlike wild type tissues (**Fig. 4B**). This is in line with previous observation seen in Fetuin A knockout mice generated using embryonic stem (ES) cells (21). Transgene was found to be inherited further from G1 to G2 progeny (**SupplementaryTable. 3**).

In conclusion, we have successfully established a novel and simple method for generating transgenic mice over expressing or knocking-down specific genes ubiquitously or in a tissue specific manner. Generation of transgenic mice showing transgene expression in infant specific Sertoli cells indicated capability of this technique to generate transgenic mice using developmentally important genes for evaluating their age specific functions. In comparison to traditionally available techniques of pronuclear DNA microinjection, retroviral gene transfer, ES cell mediated gene transfer or recent technique of testicular electroporation of transgenes using electric current, the newly developed technique of *in vivo* testicular transfection via hypotonic shock for generation of transgenic animals is very appealing. About 55% of progeny born were transgenic (**SupplementaryTable. 4**) suggesting that this technique is superior than previously published method of testicular electroporation of transgenes which uses electric current (22) and other conventional methods of generation of transgenic animal (5). Such success rate indicates that a large number of transgenic animals can be generated within a short period of time. This procedure is fast and is also easily adaptable by biomedical researchers working in various fields as it does not require any dedicated laboratory or equipment or specialized expertise for performing surgery and/or embryo manipulation. Moreover, it does not involve sacrifice of any

294 animal or use of electric pulses for testicular gene transfer making this technique ethically more
295 acceptable. This minimally-invasive technique will definitely broaden the scope of common
296 biomedical researchers in generating their own transgenic animals which will potentially
297 enhance the pace of studies involving functional genomics.

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Competing Interests:

The authors declare that they have no competing interests.

Author Contribution:

The first and second authors contributed equally to this work. The experiments were conceived and designed by SSM and AU. Experiments were performed by AU, NG1 (Nirmalya Ganguli), NG2 (Nilanjana Ganguli), RS, MC, HS. The data presented in the manuscript were analysed by all authors. The manuscript was written by AU, NG1 and SSM.

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384 **Figure Legends:**

385

386 **Fig. 1: Gene expression and cytoarchitecture of transfected testis.**

387 **(A)** Photomicrograph showing EGFP expression in fore-founder testis transfected with pCX-
388 *Egfp* suspended in 150mM Tris-HCl. **a) & b):** EGFP expression was observed mostly in germ
389 cells (yellow arrow head) of fore-founder mice. **c) & d):** Lack of EGFP expression in wild type
390 testis. **Note:** Non-specific signal (red arrow head) in the interstitial space of the testis was
391 observed in both transgenic and non-transgenic animal's tissue section. Scale bar: 50 µm.
392 **(B)** Seminiferous tubule showing EGFP in germ cells at higher magnification. Scale bar: 10 µm.
393 **(C)** Shows normalcy of testicular architecture of the fore founder mice testis, staining with
394 hematoxylin and eosin. Scale bar: 50 µm.

395

396 **Fig. 2: Validation of *Amh-Ires2-Egfp* transgenic mice.**

397 **(A)** Southern Blot analysis of genomic DNA isolated from tail biopsy of *Amh-IRES2-Egfp*
398 transgenic mice, showing integration of transgene in multiple sites. Lanes, 1 and 2 loaded with
399 gDNA of wild type FVB/J mice, 3, 4, 5 and 6 loaded with gDNA of transgene bearing FVB/J
400 mice. L denotes 1kb DNA ladder (NEB, USA).
401 **(B)** EGFP expression in Sertoli cells of 5 days old *Amh-IRES2-Egfp* transgenic mice. **a)** EGFP
402 expression was observed specifically in Sertoli cells of the testis. **b**) Corresponding merged
403 image with DAPI. Yellow arrow head shows the EGFP flourescence in the Sertoli cells inside
404 seminiferous tubules. **c) & d)** shows EGFP expression in seminiferous tubule in higher
405 magnification. **e) & f)** Testis section of wild type mice. Scale bar: a - f 10µm. White Arrow
406 marks the nonspecific staining in Leydig Cells.

(C) Western blot analysis of protein isolated from testis of 5 days old transgenic as well as wild-type mice. One G1 progeny (MT22) and one G2 progeny (MT43) showed EGFP expression (~28 kDa band). β-actin was used as internal control.

Fig. 3: Validation of *Bucsn2-IRES2-Egfp* transgenic mice.

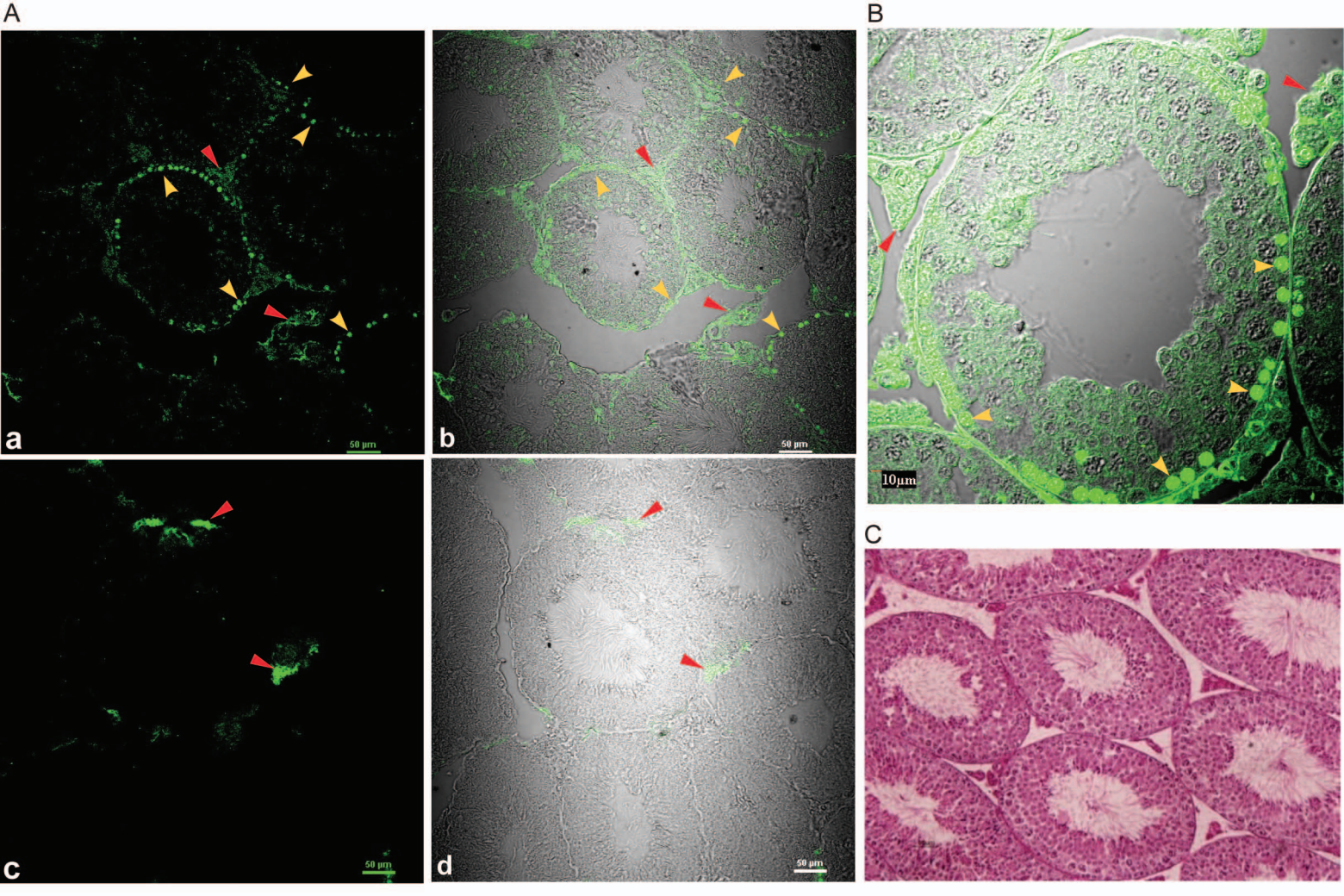
(A) Mammary gland of lactating (day 7 of lactation) transgenic female mice (TBc 7) carrying *Bucsn2-IRES2-Egfp* transgene, as observed under stereozoom fluorescence microscope. Image (i) show the EGFP fluorescence specifically in mammary gland. Image (iii) show no EGFP fluorescence in gland of wild type mice. Image (ii) and (iv): corresponding phase contrast image.

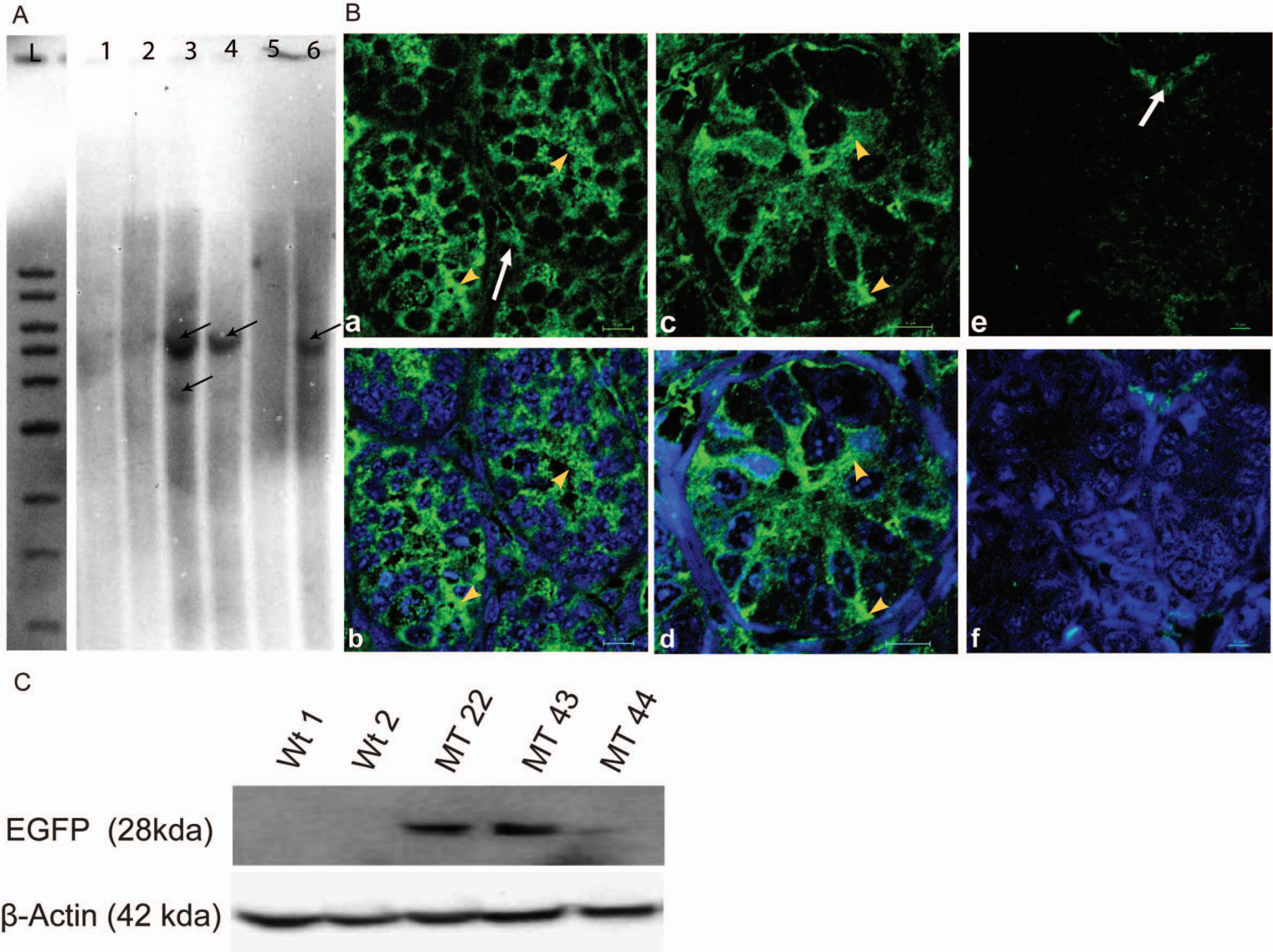
(B) Western blot analysis of protein isolated from various tissues (liver, spleen, brain, heart, mammary gland & kidney) of lactating (day 7 of lactation) transgenic female mice (TBc 7) carrying *Bucsn2-IRES2-Egfp* transgene. GFP expression (~28 kDa) was present only in mammary gland sample and not in other tissue type of the animal. β-actin was used as loading control.

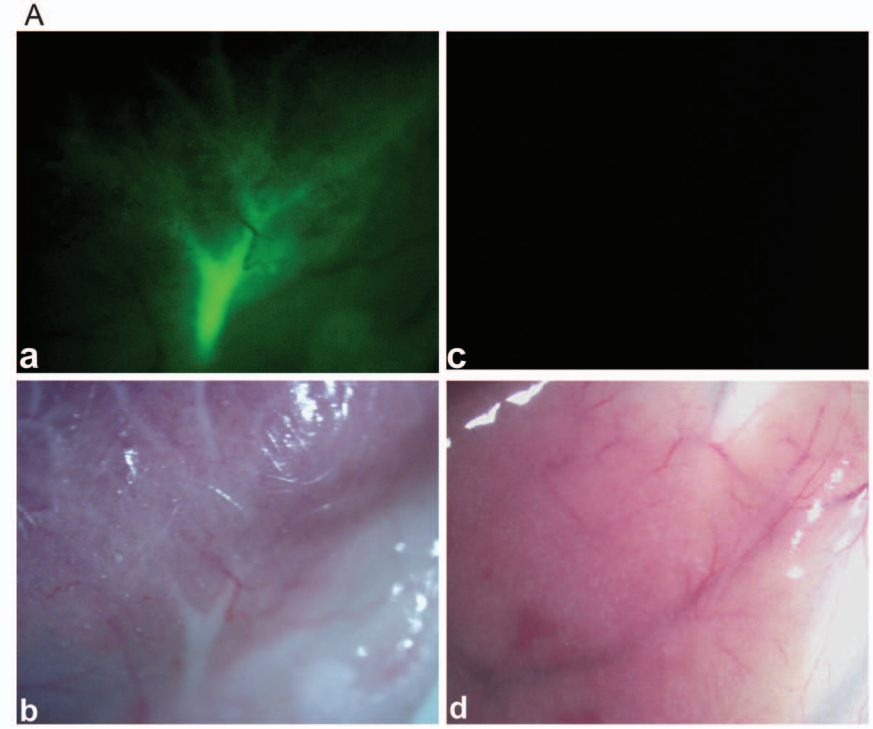
Fig. 4: Evaluation of Fetuin-A knockdown in mice.

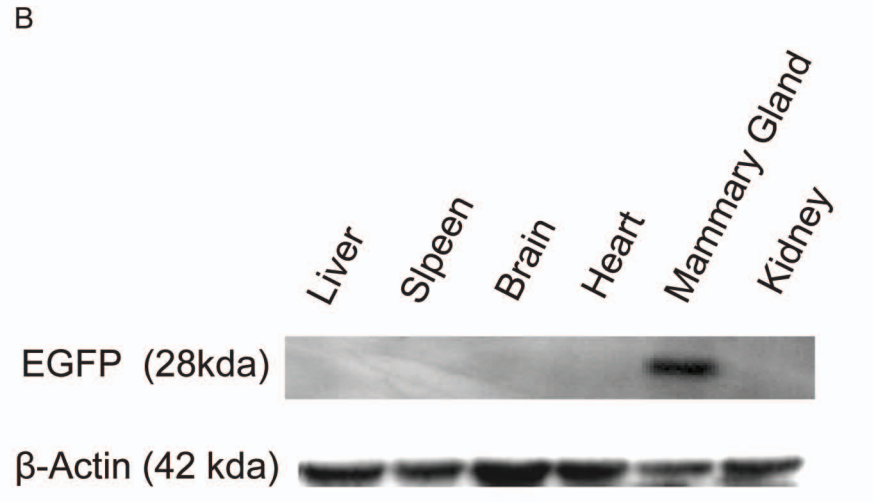
(A) Relative fold changes in Fetuin-A mRNA expression of transgenic animals relative to wild type animals. Grey bars (FT10 - FT15) represent G1 transgenic animal bearing shRNA against Fetuin-A transcript. White bar represents wild type animals (n=3). *** pValue<0.01.

(B) Von kossa staining for detection of increased calcium deposition (black spots) in Fetuin A knockdown mice. **a) & b)** cross section of liver; **c) & d)** cross section of heart.

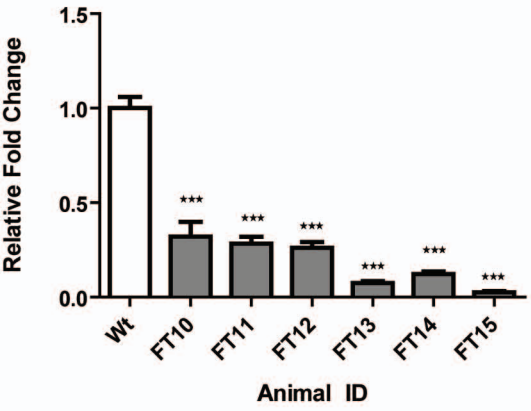

A
B
C
a
b
c
d
50 μm
50 μm
50 μm
50 μm
10μm


A
L
1
2
3
4
5
6
B
a
b
c
d
e
f
C
Wt 1
Wt 2
MT 22
MT 43
MT 44
EGFP (28kda)
β-Actin (42 kda)

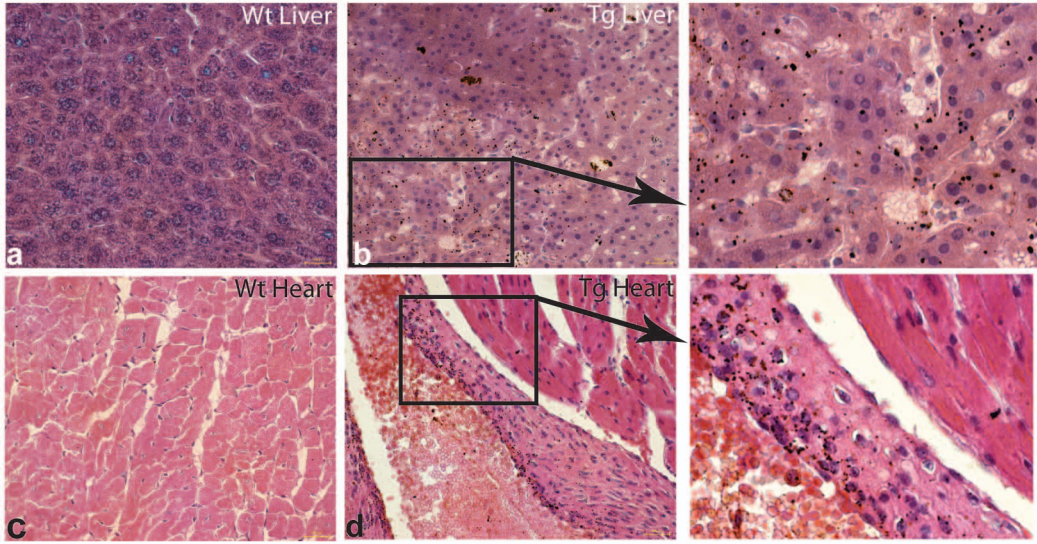

A
a
c
b
d


B
Liver
Slpeen
Brain
Heart
Mammary Gland
Kidney
EGFP (28kda)
β-Actin (42 kda)

A


Relative Fold Change
1.5
1.0
0.5
0.0
***
***
***
***
***
***
Wt
FT10
FT11
FT12
FT13
FT14
FT15
Animal ID

B


Wt Liver
Tg Liver
a
b
Wt Heart
Tg Heart
c
d