

## CHAPTER 3: RESULTS

### 3.1. Transcriptional regulation of TFF1 by E<sub>2</sub> *in vitro*

#### 3.1.1. E<sub>2</sub> was able to enhance the TFF1 transcription

To confirm that human TFF1 is transcriptionally regulated in a dose-dependent manner by E<sub>2</sub> *in vitro*, MCF-7 cells were exposed for 24 h to different concentrations of E<sub>2</sub> (10<sup>-15</sup>, 10<sup>-8</sup>, 10<sup>-5</sup> M), ICI 182 780 (10<sup>-7</sup> M), tamoxifen (10<sup>-6</sup> M), and combinations of these substances as indicated in table 4.

RT-PCR and quantitative real-time PCR revealed that different concentrations of estradiol (10<sup>-15</sup>, 10<sup>-8</sup>, 10<sup>-5</sup> M) were able to significantly enhance the transcription of TFF1 in MCF-7 cells (Fig. 8A and B), with a maximal 3.8-fold increase of TFF1 mRNA ( $0.138 \pm 0.037$ ) at a concentration of 10<sup>-15</sup> M when compared to the control ( $0.036 \pm 0.019$ ), whereas at a concentration of 10<sup>-8</sup> M, estradiol was able to maximally upregulate the expression of TFF1 at protein level (Fig. 8C). However, unexpectedly, it seems that ICI 182 780 and tamoxifen were each able to significantly enhance the transcription of TFF1, with a 2.6-fold increase by ICI 182 780 at 10<sup>-7</sup> M ( $0.092 \pm 0.0039$ ) and a 7.6-fold increase by tamoxifen at 10<sup>-6</sup> M ( $0.274 \pm 0.061$ ) when compared to the control ( $0.036 \pm 0.019$ ) (Fig. 8A and B). Furthermore, it seems that 10<sup>-6</sup> M tamoxifen was more potent to activate the TFF1 transcription than 10<sup>-15</sup>, 10<sup>-8</sup> and 10<sup>-5</sup> M estradiol (Fig. 8B). However, in the presence of estradiol, ICI 182 780 and tamoxifen appeared to inactivate the TFF1 transcription, because the TFF1 transcriptional level was reduced 6.8-fold in the cells treated with 10<sup>-7</sup> M ICI 182 780 plus 10<sup>-15</sup> M estradiol ( $0.02 \pm 0.0035$ ), and 7.4-fold reduced in the cells treated with 10<sup>-6</sup> M tamoxifen plus 10<sup>-15</sup> M estradiol ( $0.0187 \pm 0.0009$ ) when compared to the transcriptional level in the cells treated with 10<sup>-15</sup> M estradiol alone ( $0.02 \pm 0.0035$ ) (Fig. 8A and B). The same results were also observed in 10<sup>-8</sup> M and 10<sup>-5</sup> M estradiol-treated groups (Fig. 8A and B).

**Table 4. Treatment scheme of MCF-7 with E<sub>2</sub>, ICI 182 780 and tamoxifen**

Group	Ethanol	E <sub>2</sub> (M)	ICI 182 780 (M)	Tamoxifen(M)
1	-	-	-	-
2	2ul	-	-	-
3	1ul	-	10 <sup>-7</sup>	-
4	1ul	-	-	10 <sup>-6</sup>
5	1ul	10 <sup>-15</sup>	-	-
6	-	10 <sup>-15</sup>	10 <sup>-7</sup>	-
7	1ul	10 <sup>-15</sup>	-	10 <sup>-6</sup>
8	1ul	10 <sup>-8</sup>	-	-
9	-	10 <sup>-8</sup>	10 <sup>-7</sup>	-
10	-	10 <sup>-8</sup>	-	10 <sup>-6</sup>
11	1ul	10 <sup>-5</sup>	-	-
12	-	10 <sup>-5</sup>	10 <sup>-7</sup>	-
13	-	10 <sup>-5</sup>	-	10 <sup>-6</sup>

ICI 182 780 and tamoxifen were dissolved in ethanol.

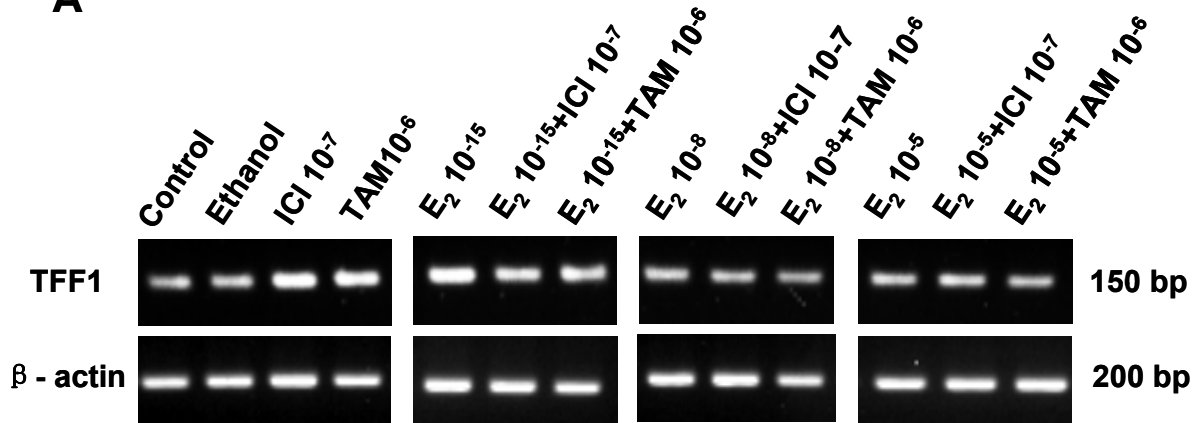
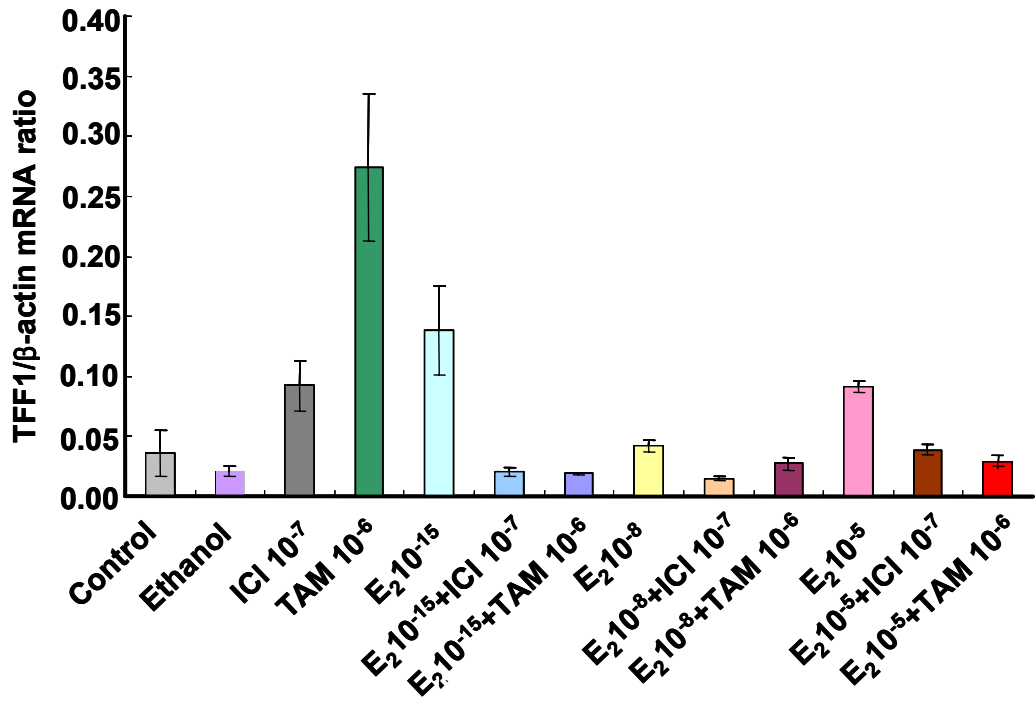
### **3. 1. 2. 10<sup>-15</sup> M estradiol upregulated the TFF1 expression, whereas 10<sup>-5</sup> M estradiol downregulated its expression.**

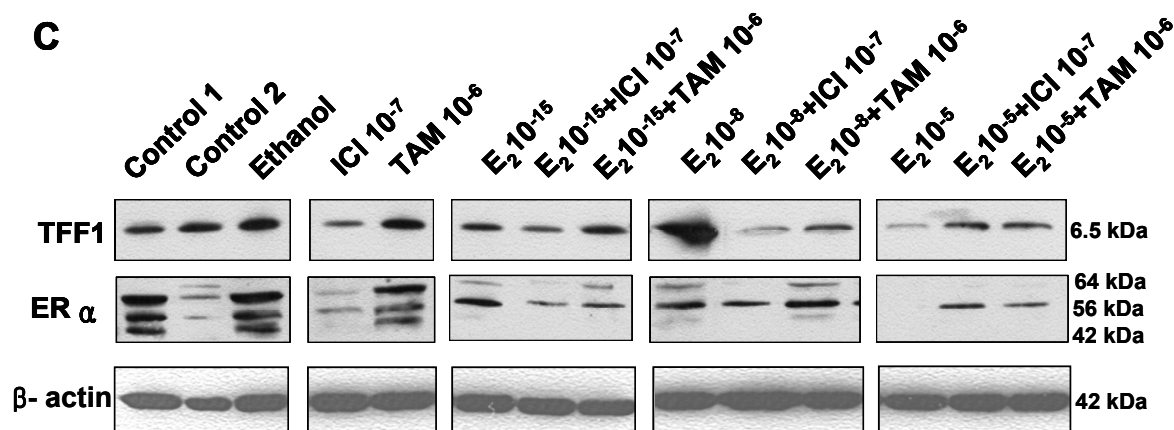
At protein level, however, estradiol, ICI 182 780 and tamoxifen had different effects on TFF1 expression. It appeared that 10<sup>-7</sup> M ICI 182 780 alone was able to downregulate TFF1 expression when compared to the control group (Fig. 8C), which was different from its agonistic effect on TFF1 transcription. In contrast, 10<sup>-6</sup> M tamoxifen alone was still able to upregulate the TFF1 expression (Fig. 8C), but the upregulation of TFF1 expression at protein level was not so high as at mRNA level. However, it appeared that low concentrations of E<sub>2</sub> (10<sup>-15</sup>M) were not able to significantly upregulate TFF1 expression. Instead, 10<sup>-8</sup> M estradiol was able to maximally upregulate the TFF1 expression. However when the estradiol concentration increased to 10<sup>-5</sup> M, the estradiol was not able to upregulate the TFF1 expression. In fact, 10<sup>-5</sup> M estradiol significantly downregulated the TFF1 expression when compared to the control (Fig. 8C). These results indicated that different concentrations of estradiol might exert different effects on TFF1 expression.

However, in the presence of ICI 182 780, the agonistic effect of estradiol on the TFF1 expression was significantly antagonized at protein level.

### **3. 1. 3. Estradiol, ICI 182 780 and tamoxifen regulated the TFF1 expression by downregulation of ER $\alpha$ expression in cytosol.**

To investigate whether ER $\alpha$  was involved in the transcriptional regulation of TFF1 by estradiol, ICI 182 780 and tamoxifen, western blot was performed to quantify the cytosolic ER $\alpha$ . Three bands of ER $\alpha$  were observed at 64, 56, 42 kDa in the control group cells. The shorter bands (56 and 42 kDa) were derived from the alternative usage of initiation ATG or splicing of ER $\alpha$  (Friend et al., 1995; Friend et al., 1997). The specificity of the immunoreactivity was tested by using peptide-preabsorbed antibodies and substituting TBS instead of primary antibody for ER $\alpha$  (Schonfelder et al., 2004). It was shown that in the control cells, a large amount of ER $\alpha$  was found in the cytosol. However, in the cells treated with either estradiol or ICI 182 780, or tamoxifen, the ER $\alpha$  expression (64, 56, and 42 kDa) was prominently downregulated in the cytosol when compared to the control cells (Fig. 8C). And the ER $\alpha$  expression in the cytosol was further downregulated in the cells treated with estradiol plus ICI 182 780 (Fig. 8C). The same result was observed in the cells treated with estradiol plus tamoxifen (Fig. 8C). Furthermore, only two bands of ER $\alpha$  variant at 64 and 56 kDa were observed in the cells treated with estradiol ( $10^{-15}$ , or  $10^{-8}$  M) plus with  $10^{-7}$  M ICI 182 780. The same result was observed in the cells treated with estradiol ( $10^{-15}$ , or  $10^{-8}$  M) plus with  $10^{-6}$  M tamoxifen, (Fig. 8C). These observations indicated that ER $\alpha$  might migrate from the cytoplasm to the nucleus to facilitate the transcriptional regulation of TFF1 during the transcriptional process, or ER $\alpha$  was estradiol-dependently degraded by the proteasome described by Nawaz (Nawaz et al., 1999). Interestingly, only one band of ER $\alpha$  variant at 56 kDa was observed in the cells treated with  $10^{-5}$  M estradiol plus  $10^{-7}$  M ICI 182 780, the same result was observed in the cells treated with  $10^{-5}$  M estradiol plus  $10^{-6}$  M tamoxifen. Furthermore, no band of ER $\alpha$  was observed in the cells treated with  $10^{-5}$  M estradiol (Fig. 8C).

**A****B**



**FIG. 8. Transcriptional regulation of TFF1 by estradiol, tamoxifen, and ICI 182 780 in MCF-7 cells**

Estradiol, ICI 182 780, and tamoxifen were each able to enhance the transcription of TFF1 by downregulation of ER $\alpha$  expression in cytosol. MCF-7 cells were treated for 24 h with different concentrations of estradiol (10<sup>-15</sup>, 10<sup>-8</sup>, 10<sup>-5</sup> M) and ICI 182 780 (10<sup>-7</sup> M) and tamoxifen (10<sup>-6</sup> M). (A) Representative RT-PCR analysis; (B) Representative quantitative real-time PCR analysis; (C) Representative western blot analysis: protein loading was normalized to  $\beta$ -actin using a monoclonal primary antibody at a 1:10,000 dilution. TAM: tamoxifen; ICI: ICI 182 780; Control1: cells cultured in phenol red free media; Control2: cells cultured in media with phenol red.

### **3. 1. 4. Estradiol and ICI 182 780 downregulated ER $\alpha$ expression, but tamoxifen upregulated ER $\alpha$ expression in nucleus.**

Confocal microscopy analysis was performed to further investigate whether the downregulated ER $\alpha$  expression in the cytosol is due to the translocation of ER $\alpha$  from the cytoplasm to the nucleus, or due to the estradiol-dependent degradation of ER $\alpha$  by the proteasome (Nawaz et al., 1999). This analysis was also used to investigate the relationship between TFF1 and ER $\alpha$  during the transcription by estradiol, ICI 182 780, and tamoxifen. It was demonstrated that ER $\alpha$  was mainly expressed in the nucleus of MCF-7 cells, which is indicated in red (Fig.9), while TFF1 was expressed in the cytoplasm with a preferential perinuclear accumulation, which is indicated in green (Fig.9). Our

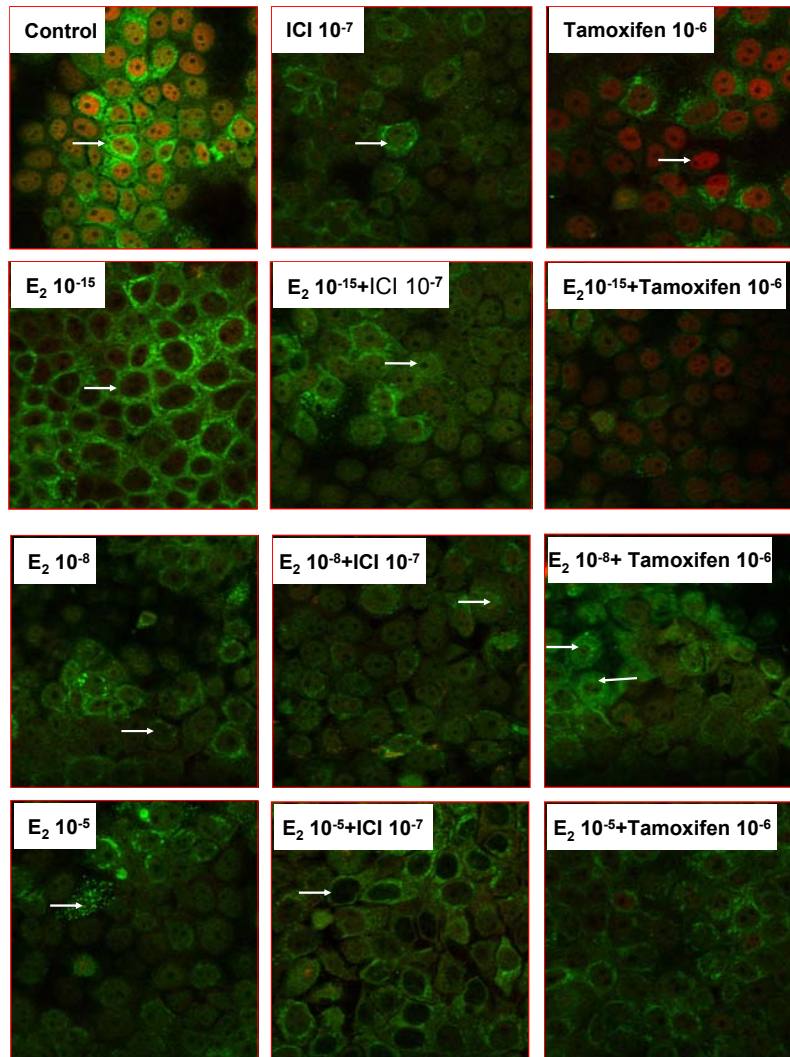
results indicated that a very low estradiol concentration ( $10^{-15}$  M) was able to upregulate TFF1 expression in the cytoplasm, which is in line with previous reports (Rio and Chambon, 1990; Lefebvre et al., 1993). We also showed that in the untreated cells, ER $\alpha$  was mainly expressed in the nuclei (Fig.9). However, after treating with different concentrations of estradiol, the number of ER $\alpha$  positive cells decreased. Furthermore, the intensity of the labeling of ER $\alpha$  in the nuclei significantly decreased when compared with control cells (Fig. 9), suggesting the ER $\alpha$  expression in the nuclei was decreased.

ICI 182 780 had the same effect as estradiol on the ER $\alpha$  expression. Treating the cells with ICI 182 780 led to a decrease in ER $\alpha$  positive cells and reduced intensity of the labeling ER $\alpha$  in nuclei. However, treating the cells with tamoxifen resulted in an increase of ER $\alpha$  positive cell number and increased intensity of the labeling of ER $\alpha$  in nuclei (Fig. 9), namely, the increased ER $\alpha$  expression in the nuclei.

All these results suggested that estradiol and ICI 182 780 exert their transcription regulating function on TFF1 through translocation of ER $\alpha$  to the nucleus where the ER $\alpha$  was degraded by the proteosome in nuclei. However, tamoxifen appeared to trigger the translocation of ER $\alpha$  to nuclei where the ER $\alpha$  was not degraded.

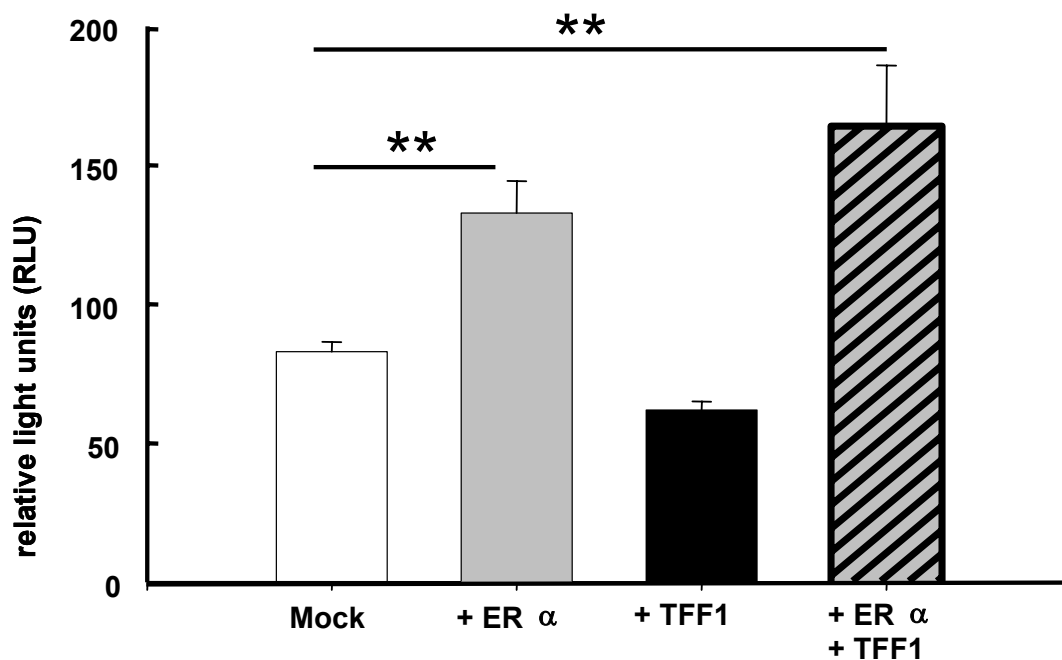
### **3. 1. 5. TFF1 activated the E-cadherin promoter in the presence of estradiol**

To investigate whether TFF1 can interact with the E-cadherin promoter, reporter assay was performed. For this purpose, MCF-7 cells were transiently co-transfected with E-cadherin promoter plasmid, TFF1-expressing plasmid, and ER $\alpha$ -expressing vector. The reporter assay revealed that ER $\alpha$  was able to activate the E-cadherin promoter, whereas TFF1 alone decreased the E-cadherin promoter activity. However, in the presence of ER $\alpha$ , TFF1 was able to activate the E-cadherin promoter (Fig. 10).



**FIG. 9. Confocal microscopy analysis of the localization of TFF1 and ER $\alpha$  in MCF-7 cells treated with different concentrations of estradiol, ICI 182 780 and tamoxifen**

Estradiol, and ICI 182 780 downregulated ER $\alpha$  expression in the nucleus, but tamoxifen upregulated ER $\alpha$  expression in nucleus of MCF-7 cells. After MCF-7 cells were treated for 24 h with different concentrations of estradiol (10<sup>-15</sup>, 10<sup>-8</sup>, 10<sup>-5</sup> M), ICI 182 780 (10<sup>-7</sup> M) and Tamoxifen (10<sup>-6</sup> M), the cells were fixed with pH 7.4 paraformaldehyde and incubated with 2.5% mouse serum. Next, the primary antibodies mouse anti-human TFF1 and rabbit anti-human ER $\alpha$  were added and incubated at 4°C overnight. The binding of primary antibodies was detected with FITC-conjugated polyclonal goat anti-mouse antibody and TR-conjugated goat anti-rabbit antibody. Cells were washed and slides were mounted in vectashield and were viewed through a confocal microscope.



**FIG.10. The effect of TFF1 on the E-cadherin promoter activity in the presence of ER $\alpha$**

Transient transfection assay revealed the activation of E-cadherin promoter by TFF1 in the presence of ER $\alpha$ . MCF-7 cells were co-transfected with the E-cadherin promoter plasmid, TFF1-expressing plasmid and an ER $\alpha$ -expressing vector. A pSV  $\beta$  galactosidase plasmid was co-transfected as an internal control. Luciferase activity was measured and normalized against the  $\beta$ -galactosidase activity. U-test was used for statistical analysis. \*\* indicates  $p < 0.01$  compared with mock group.

### 3. 2. *In vivo* experiments I

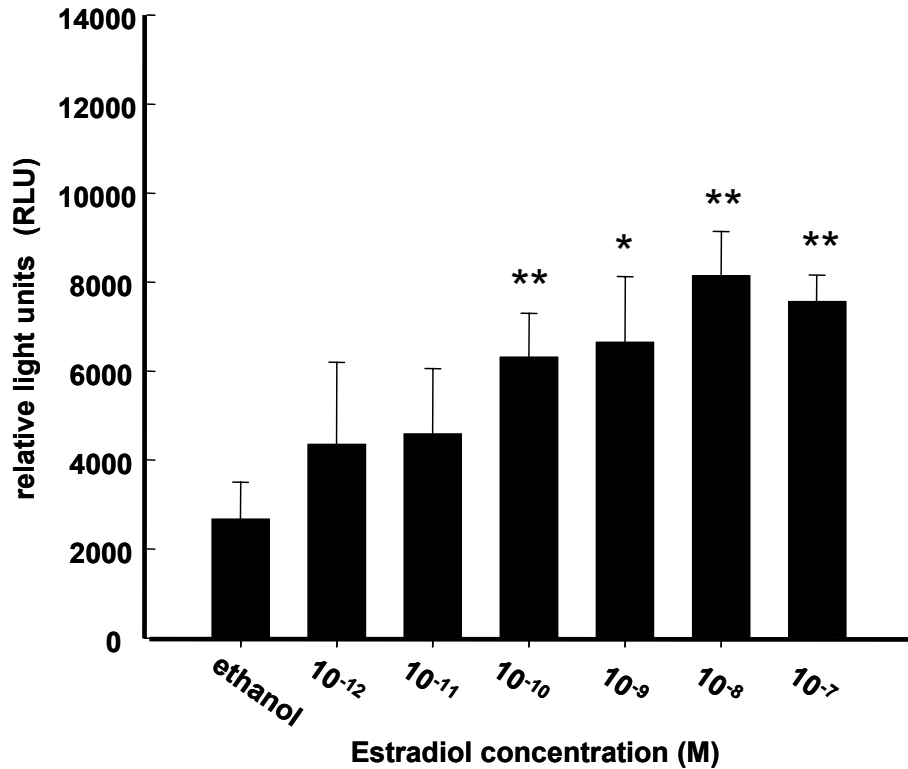
#### 3. 2.1. Generation and identification of *hTFF1-luc* transgenic rats

##### 3. 2.1.1. Establishment of the *hTFF1-pGL3* construct

To generate an *hTFF1-luc* transgenic rat line, the human TFF1 promoter (1000 bp from the 5' flanking region) was first cloned upstream of the luciferase cDNA into the pGL-3 plasmid. To examine whether the new modified *hTFF1-pGL3* plasmid was able to respond to estradiol, the *hTFF1-pGL3* plasmid was transiently transfected into MCF-7 cells as described in the materials and methods section. *In vitro* luciferase activity assay



revealed that estradiol (from  $10^{-10}$  to  $10^{-7}$  M) was able to activate the hTFF1 promoter (Fig. 11), suggesting that the hTFF1-pGL-3 vector was successfully established.

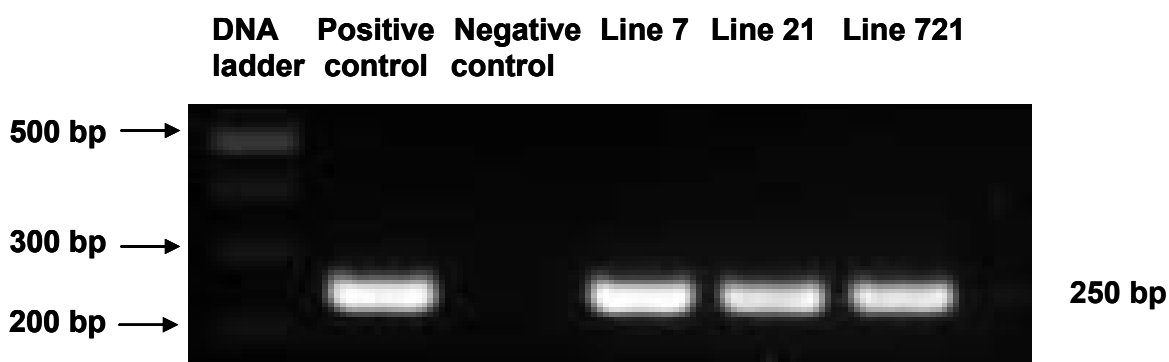


**FIG.11. Transcriptional regulation of hTFF1 promoter in hTFF1-pGL3 construct by estradiol**

Transient transfection assay revealed that the new modified hTFF1-pGL3 construct could be activated by estradiol. After MCF-7 cells were cultured in estrogen-depleted medium for 3 days, the plasmid of hTFF1-pGL3 was transfected with control plasmid pSV  $\beta$ -galactosidase as described in the materials and methods section. The day following the transfection, the transient-transfected cells were treated for 24 h with a serial concentration of estradiol. Luciferase and  $\beta$ -galactosidase activity assays were performed, and the luciferase activities were normalized to  $\beta$ -galactosidase activities. U-test was used for statistical analysis.  $n=3$ . \* indicates  $p<0.05$ , \*\* indicates  $p<0.01$  compared with vehicle (ethanol) treated cells.

### 3. 2. 1. 2. *hTFF1-luc* transgenic rat lines

Three transgenic founder rats (line 7, line 21 and line 721) were obtained (Fig. 12), and all lines were fertile. The offspring from each line were screened for presence of the transgene by PCR analysis using luciferase-specific primers. The heterozygous (*hTFF1-luc*<sup>+/-</sup>) transgenic rats and homozygous rats (*hTFF1-luc*<sup>+/+</sup>) from each line were generated as described in the materials and methods section. Since the other transgenic rat line 21 and line 721 displayed almost the same expression results of the transgene as line 7, the transgenic rats of line 7 were used for the following experiments.



**FIG.12. Screening of *hTFF1-luc* transgenic rat founders by PCR.**

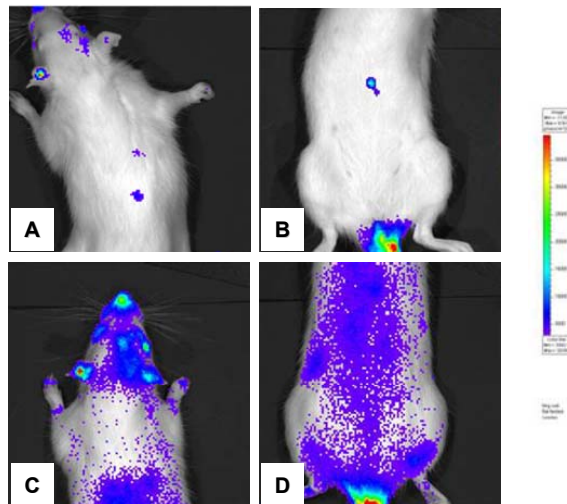
Genomic DNA was isolated from the tails of transgenic rat founders for PCR analysis to detect the presence of the luciferase transgene. The PCR reaction generated a 250 bp PCR product. The *hTFF1-pGL3* plasmid served as positive control. Aliquots of PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by staining with ethidium bromide. Three transgenic founder rats (line 7, line 21 and line 721) were established.

### 3. 2. 1. 3. *In vivo* bioluminescence imaging of the *hTFF1-luc* transgene in living animals

The *hTFF1-luc*<sup>+/-</sup> transgenic female rats of line 7 were further analyzed for the luciferase transgene expression fashion in organs by *in vivo* bioluminescence imaging after intraperitoneally injecting luciferin. Serial images were obtained from the rats at different

times after injecting the luciferin, and the mean photon flux relative to the peak signal was determined. The bioluminescence reflected the luciferase activity. Each result was a pseudocolor illustration overlain on a gray-scale reference image of the whole rat.

Fig. 13 shows the bioluminescence images of a representative *hTFF1-luc*<sup>+/-</sup> transgenic female rat of line 7 over a time period. *In vivo* bioluminescence imaging revealed that no or little bioluminescence was detected 5 min after injecting luciferin. However, 10 min after injection of luciferin, more bioluminescence was detected in the vagina region, and no bioluminescence was detected in the heart and stomach regions. From 10 to 52 min, the bioluminescence increased gradually, and bioluminescence was detected in brain and heart. From 52 to 107 min, the bioluminescence was eventually detected in stomach and uterus, with strongest bioluminescence in vagina. All these data suggested that the *hTFF1-luc* transgene was expressed in the vagina, brain, heart, uterus and stomach of the *hTFF1-luc*<sup>+/-</sup> transgenic rats. The highest luciferase activity was in the vagina, and some luciferase activity in heart and brain, and low luciferase activity in the uterus and stomach (Fig. 13).



**FIG.13. Identification of the *hTFF1-luc* transgene expression by *in vivo* bioluminescence imaging**

*In vivo* bioluminescence imaging revealed that the luciferase transgene was mainly expressed in the brain, heart, stomach, uterus and vagina. The highest luciferase activity was in the vagina, and some luciferase activity in heart and brain, and low luciferase activity in the uterus and stomach. The *hTFF1-luc*<sup>+/-</sup> transgenic female rats of line 7 were further analyzed for the luciferase transgene expression after intraperitoneal injection of luciferin. *In vivo* bioluminescence imaging of the dorsal area of female *hTFF1-luc*<sup>+/-</sup> transgenic rats was performed during the time course after injecting luciferin. Luciferase activity was detected using *in vivo* bioluminescence imaging and evaluated in different organs in the transgenic rats at 5, 10, 53 and 107 min after intraperitoneally injecting the enzyme substrate, luciferin. *In vivo* transgenic luciferase expression was assessed using low light imaging systems to measure photons that were emitted after the luciferin injection. The relative level of bioluminescence was shown as a pseudocolor display, with red representing the strongest photon fluxes, and violet representing the weakest photon fluxes.

**A:** luciferase activity at 5 min after intraperitoneal injection of luciferin.

**B:** luciferase activity at 10 min after intraperitoneal injection of luciferin, light emission was restricted to vagina region.

**C:** luciferase activity at 52 min after intraperitoneal injection of luciferin; light emission was restricted to brain, heart and stomach regions.

**D:** luciferase activity at 107 min after intraperitoneal injection of luciferin; light emission was restricted to uterus and vagina regions.

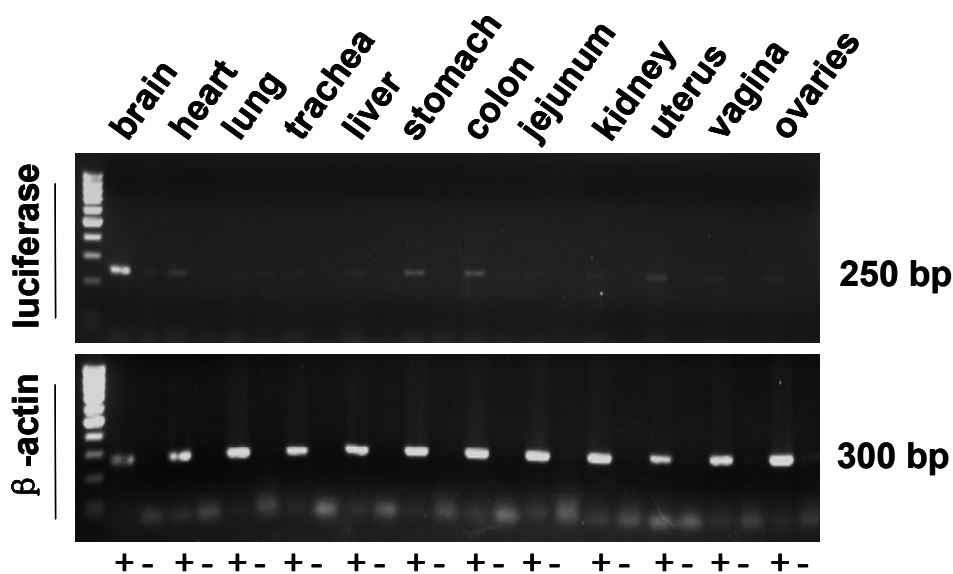
### **3. 2. 2. Tissue expression fashion of luciferase transgene**

#### **3. 2. 2. 1. Luciferase transgene was highly expressed in brain, followed by heart, stomach, colon, uterus, vagina and ovary.**

To precisely examine whether this hTFF1 promoter was capable of directing tissue-specific expression of luciferase transgene, a variety of tissues from 4-month-old female *hTFF1-luc<sup>+/-</sup>* transgenic rats at estrous stage from line 7 were analyzed for luciferase expression. RT-PCR analysis, using luciferase-specific primers, revealed that the luciferase transgene was detected in the tissues of brain, heart, stomach, colon, uterus, vagina and ovary (Fig. 14). This indicated that the hTFF1 promoter in these tissues was activated, but it was not activated in the tissues of lung, trachea, liver, jejunum and kidney. The tissue expression of luciferase was quite similar to that of the endogenous TFF1 tissue expression fashion (Hirota et al., 1994; Masui et al., 2006; Wiede et al., 2001). These results suggested the integration of the luciferase gene into the rat genome, and the expression of luciferase transgene can be directed by the human TFF1 promoter in a tissue-specific fashion.

Among the luciferase expressing organs, the luciferase mRNA expression level was highest in the brain, suggesting the hTFF1 promoter was highly activated within the brain of the *hTFF1-luc<sup>+/-</sup>* transgenic rat at estrous stage. The high basal level of luciferase activity in brain might result from the exposure of hTFF1 promoter to brain-specific proteins that serve as transcriptional factors of the hTFF1 promoter.

These results agreed with the data obtained by *in vivo* bioluminescence imaging of the luciferase expression. Both methods detected luciferase expression in the brain, heart, stomach, uterus and vagina.



**FIG. 14. RT-PCR analysis of the *hTFF1-luc* tissue expression pattern in female *hTFF1-luc*<sup>+/-</sup> transgenic rats at estrous stage**

RT-PCR revealed that the luciferase transgene was expressed in the tissues of brain, heart, stomach, colon, uterus, vagina and ovary, with highest expression in the brain. Total RNA was isolated and purified from various tissues of *hTFF1-luc*<sup>+/-</sup> female rats. RT-PCR detection of luciferase was performed with luciferase-specific primers as described in the materials and methods section. PCR products were analyzed by electrophoresis on an 2% agarose gel and visualized by staining with ethidium bromide. The expected size of luciferase-derived PCR product is about 250 bp.

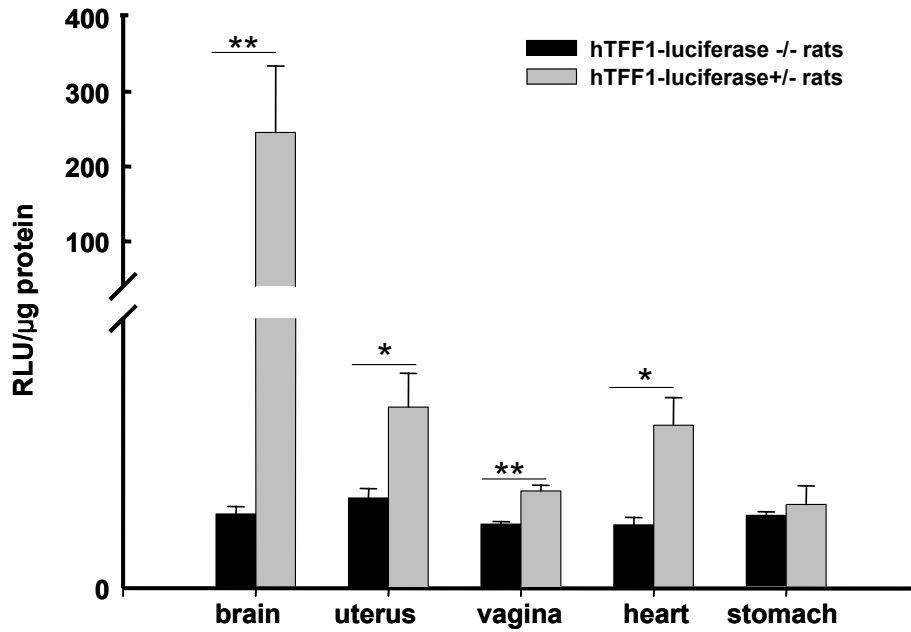
### **3. 2. 2. 2. *In vitro* luciferase activity assay confirmed the highest luciferase activity in brain, not in vagina.**

Analysis of *in vitro* luciferase activity in different tissues is considered as a good indicator of the integration of *hTFF1-luc* gene, and of the actions of ligand toward activation of the hTFF1 promoter. Furthermore, to precisely identify the highest luciferase-expressing organ in the transgenic rats, luciferase activity assay *in vitro* was performed.

Since the tissue-specific expression fashion of the *hTFF1-luc* gene had been identified by RT-PCR, only the tissues that interested us were selected again for the analysis of luciferase activity *in vitro*. We selected the tissues for a further *in vitro* luciferase activity

assay based on the following reasons. First, estrogen is shown to be able to regulate the expression of TFF1 gene (Jakowlew et al., 1984), and ER is abundantly expressed in heart (Nilsson et al., 2001). Therefore, heart was selected for analysis of the luciferase activity. Furthermore, one of the purposes for the establishment of the transgenic rat model was to investigate the transcriptional regulation and function of the TFF1 in female reproductive system. Thus, the uterus and vagina were selected to examine the luciferase activity *in vitro*. The brain and vagina were selected for luciferase activity assay to further confirm which organ expressed the highest luciferase activity. Stomach was selected because endogenous TFF1 is mainly expressed in stomach tissue. Therefore, the tissues of brain, heart, stomach, uterus and vagina were selected for analysis of luciferase activity *in vitro* and for further investigation of its transcriptional regulation by estrogen.

4-month-old *hTFF1-luc*<sup>+/-</sup> transgenic rats, from line 7 at estrous stage, combined with the wild type (*hTFF1-luc*<sup>-/-</sup>) rats (served as control), were sacrificed, and the organs were harvested for luciferase activity measurement as described in the materials and methods section. As we expected, the basal level of luciferase activity in the tissues of stomach, heart, uterus, vagina and brain from the wild type rats was very low, but the luciferase activity in the same tissues from the *hTFF1-luc*<sup>+/-</sup> transgenic rats was significantly higher than that from the wild type rats (Fig. 15). The luciferase activity in brain was 12-fold higher than in heart and uterus, and 25-fold higher than that in stomach and vagina from the *hTFF1-luc*<sup>+/-</sup> transgenic rats at estrous stage. These results further confirmed that the highest luciferase-expressing tissue in the transgenic rats was brain, not vagina.



**FIG. 15. *In vitro* luciferase activity analysis of the tissues from the female *hTFF1-luc*<sup>+/-</sup> rats at estrous stage**

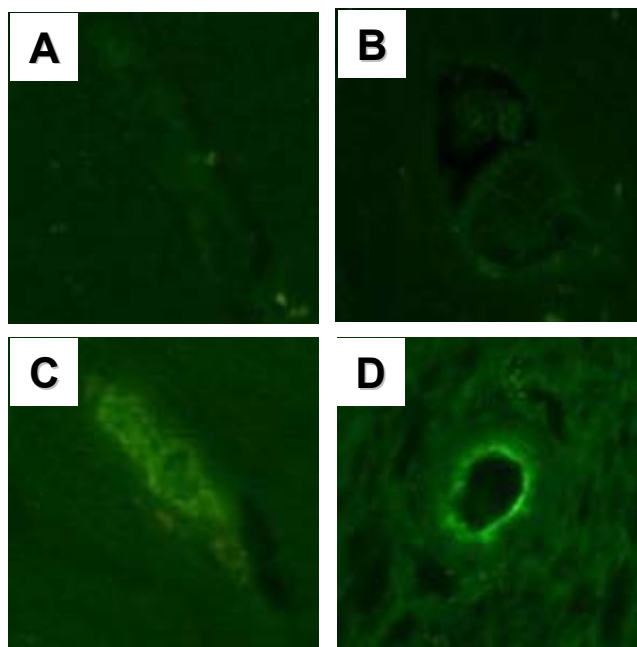
*In vitro* luciferase activity analysis revealed that luciferase was expressed in brain, uterus, vagina, heart, and stomach tissue, with the highest luciferase activity in brain. After tissues were harvested and homogenized, the protein supernatant was extracted as described in the materials and methods section. 20  $\mu$ l of the extracted supernatant was used for the measurement of relative light units (RLU) for 20 sec using a luminometer. The RLU were normalized to the protein content. Each bar was an average of luciferase activity from three different *hTFF1-luc* transgenic rats (n=3). T-test was used for statistical analysis. \*indicates  $p < 0.05$ , \*\*indicates  $p < 0.01$  compared with that in the tissues from *hTFF1-luc*<sup>-/-</sup> rats.

### 3. 2. 3. The luciferase transgene was localized in the vessel of the brain tissue.

Since the brain exhibited much higher luciferase activity compared to the other organs, the brain tissue was used to investigate the cellular localization of the luciferase gene expression in the female *hTFF1-luc*<sup>+/-</sup> transgenic rats from line 7 at estrous stage. Fig. 16 illustrates representative luciferase immunostaining plus negative control within the brain tissue from the female *hTFF1-luc*<sup>+/-</sup> transgenic rats. The green color, which indicates the



luciferase immunostaining, was detected in a vessel from the brain, but not in other areas of the brain.



**FIG.16. Immunofluorescence analysis of luciferase localization in the brain from *hTFF1-luc*<sup>+/-</sup> rats at estrous stage.**

Immunofluorescence analysis revealed that the localization of luciferase was detected in the vessel of the brain from *hTFF1-luc*<sup>+/-</sup> rats with an intense staining in green (C and D). The negative controls in the brain from *hTFF1-luc*<sup>+/-</sup> rats showed no specific luciferase staining (A and B).

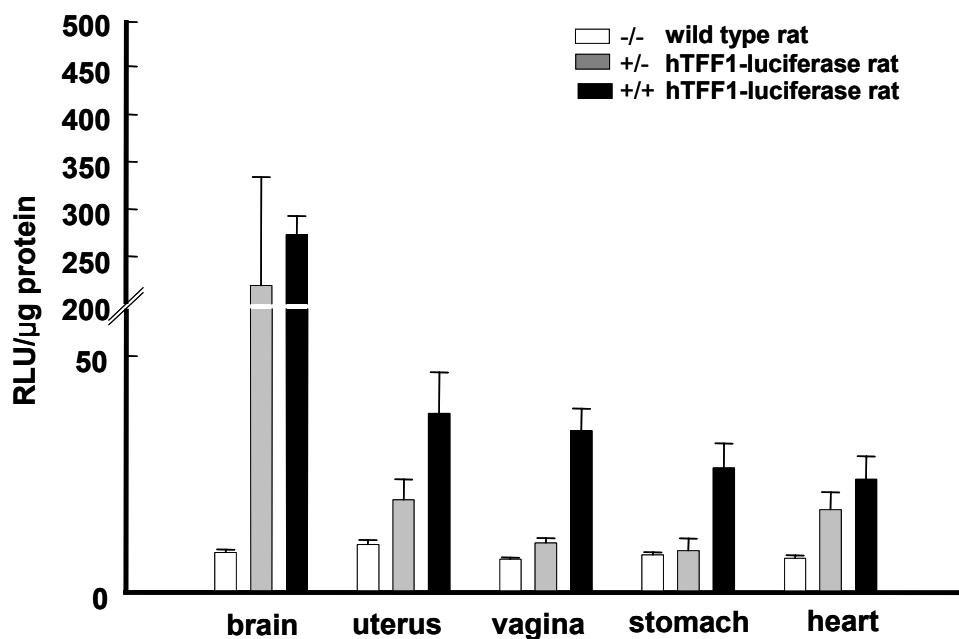
### **3. 2. 4. The hTFF1 promoter was activated by the serum estrogen during natural estrous cycle in *hTFF1-luc*<sup>+/+</sup> transgenic rats.**

#### **3. 2. 4. 1. Generation and identification of the *hTFF1-luc*<sup>+/+</sup> transgenic rats**

The *hTFF1-luc* transgenic rats were bred with wild type SD rats to generate *hTFF1-luc*<sup>+/-</sup> (heterozygous) transgenic rats. The *hTFF1-luc*<sup>+/-</sup> transgenic rats were then inbred to produce the *hTFF1-luc*<sup>+/+</sup> (homozygous) rats. We identified *hTFF1-luc*<sup>+/+</sup> transgenic rats using the classic Mendel's Equal Segregation Law after backcrossing each of the transgenic rats onto wild type SD rats. The offspring from individual matings were

analyzed by PCR for the presence of the luciferase gene. If all the offspring were luciferase positive, one of the parent from the transgenic rat was identified to be a *hTFF1-luc*<sup>+/+</sup> rat.

We further confirmed some *hTFF1-luc*<sup>+/+</sup> rats by comparing the luciferase activity in the tissues with that of the *hTFF1-luc*<sup>+/-</sup>, and wild type (*hTFF1-luc*<sup>-/-</sup>) rats. Fig.17 shows that the luciferase activity in the brain tissue from the *hTFF1-luc*<sup>+/+</sup> rats was much higher than that from *hTFF1-luc*<sup>+/-</sup> and *hTFF1-luc*<sup>-/-</sup> rats. *In vitro* luciferase activity revealed that in *hTFF1-luc*<sup>+/+</sup> rats, the luciferase activity in the heart ( $25.3 \pm 3.5$ ), stomach ( $26.4 \pm 5.1$ ), uterus ( $35.1 \pm 11.4$ ) and vagina ( $18.1 \pm 3.1$ ) was 2-3 fold higher than in the *hTFF1-luc*<sup>+/-</sup> rat heart ( $18.1 \pm 3.1$ ), stomach ( $9.3 \pm 2.1$ ), uterus ( $20.1 \pm 3.8$ ) and vagina ( $10.8 \pm 0.7$ ), which contributed to further confirmation of the *hTFF1-luc*<sup>+/+</sup> transgenic rats.



**FIG. 17. Identification of the *hTFF1-luc*<sup>+/+</sup> transgenic rats by comparison of luciferase activity in tissues**

After the tissues were harvested from the *hTFF1-luc*<sup>+/+</sup>, *hTFF1-luc*<sup>+/-</sup> and wild type (*hTFF1-luc*<sup>-/-</sup>) rats at estrous stage, they were homogenized, and the protein supernatants were extracted as described in the materials and methods section. 20  $\mu$ l of the extract supernatant was used for the measurement of

relative light units (RLU) for 20 sec using a luminometer. The RLU were normalized to the protein content. Each bar represents an average of luciferase activity from three rats at estrous (n=3).

### **3. 2. 4. 2. Luciferase activity was significantly increased in the tissues from the female *hTFF1-luc*<sup>+/+</sup> rats at proestrous stage.**

Since TFF1 gene is reported to be regulated by estrogen (Lu et al., 2001; Miyashita et al., 1994), investigating the effect of natural estrous cycle on the luciferase gene expression could reflect the integration of the *hTFF1-luc* transgene and its transcriptional regulation by serum estrogen in the transgenic rats. The female *hTFF1-luc*<sup>+/+</sup> rats were randomly selected for determination of the estrous stage. The *hTFF1-luc*<sup>+/+</sup> rats were used because they were considered to be more sensitive to the hormonal regulation due to their double luciferase transgenes on the chromosomes. The female *hTFF1-luc*<sup>+/+</sup> transgenic rats were sacrificed at a definite estrous stage. The blood was collected for serum estradiol measurement, and the tissues were excised for further analysis of luciferase activity.

First, we analyzed and compared the luciferase activity among different tissues from the *hTFF1-luc*<sup>+/+</sup> rats at estrous stage. The analysis of *in vitro* luciferase activity revealed that at estrous, luciferase activity in the brain ( $276.1 \pm 16.7$ ) was approximately 11-fold higher than that in the heart ( $25.3 \pm 3.5$ ) and stomach ( $26.4 \pm 5.1$ ). The luciferase activity in the brain was 8-fold higher than that in the uterus ( $35.1 \pm 11.4$ ) and vagina ( $33.6 \pm 5.7$ ). And the luciferase activity in cerebellum ( $493.4 \pm 32.2$ ) was approximately 19-fold higher than that in heart and stomach, and 14-fold higher than that in uterus and vagina at estrous stage (Fig. 18A). These observations suggested that the hTFF1 promoter was highly activated in cerebellum and brain. The same results were observed in the *hTFF1-luc*<sup>+/+</sup> at the diestrous stage as well. Nevertheless, at proestrous, the luciferase activity in the brain ( $860.9 \pm 131.9$ ) significantly increased and was approximately 43-fold higher than that in heart ( $20.4 \pm 3.3$ ) and stomach ( $19.9 \pm 3.2$ ), and 26-fold higher than that in uterus ( $33.7 \pm 3.2$ ) and vagina ( $32.9 \pm 4.8$ ). The luciferase activity in the cerebellum ( $1401.2 \pm 67.5$ ) significantly increased and was approximately 70-fold higher than that in heart and stomach, and 42-fold higher than that in uterus and vagina (Fig. 18A). This suggested

that at the proestrous stage, the hTFF1 promoter in the brain and cerebellum was activated higher than in the other four tissues, although the luciferase activity in the other four tissues was increased as well.

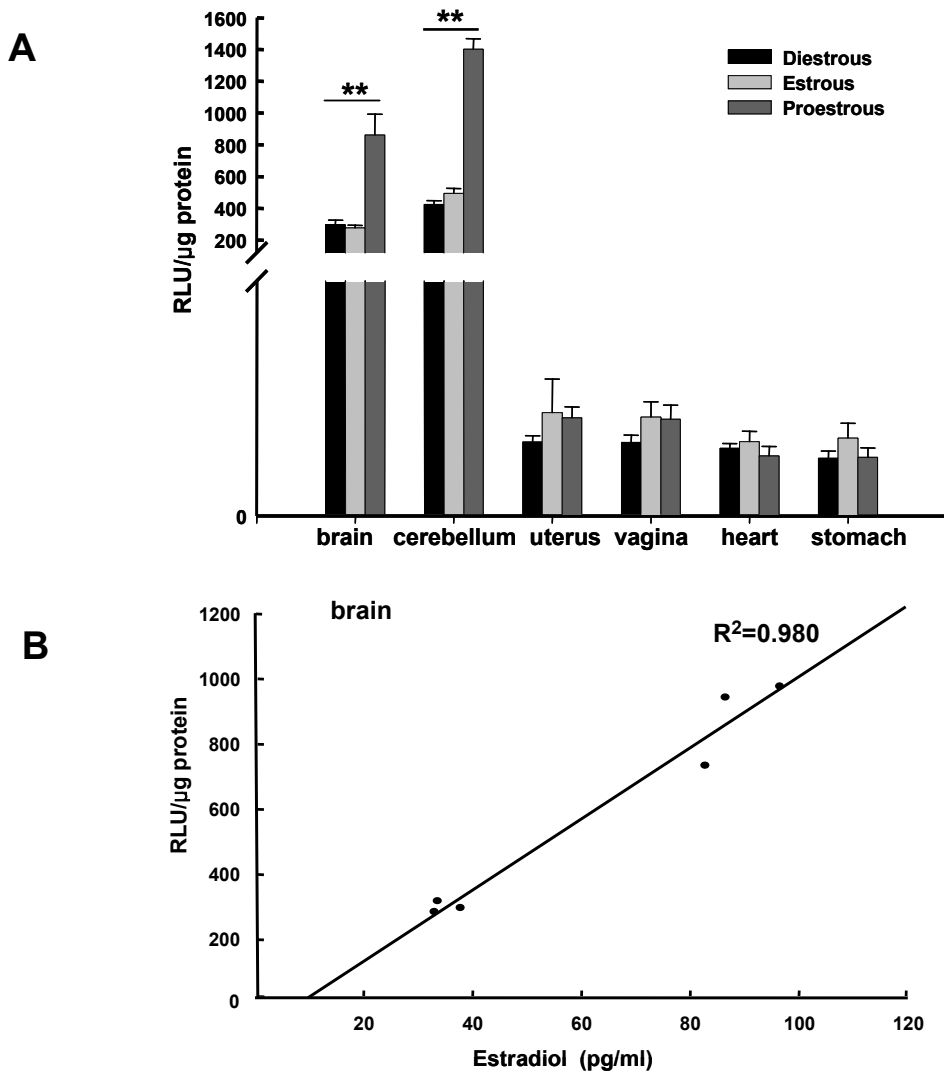
Next, we compared the luciferase activity in the same tissue from the *hTFF1-luc*<sup>+/+</sup> rats at different estrous stages. We found that, at proestrous stage, the luciferase activity in the brain ( $860.9 \pm 131.9$ ) significantly increased, and was approximately 3.7-fold higher than that in brain at the diestrous ( $296.8 \pm 28.9$ ) and estrous stages ( $276.1 \pm 16.7$ ) (Fig.18A). The luciferase activity in the cerebellum at proestrous stage ( $1401.2 \pm 67.5$ ) significantly increased as well, and was approximately 3-fold higher than that in cerebellum at the diestrous ( $423.9 \pm 24.6$ ) and estrous stages ( $493.4 \pm 32.2$ ). This observation indicated that the hTFF1 promoter in the brain and cerebellum was activated by the serum hormones at the proestrous stage. However, the luciferase activities in heart, stomach, uterus and vagina did not significantly increase at the proestrous stage, when compared to that at the diestrous and estrous stages. At the estrous stage, the luciferase activity in the six tissues increased in comparison to that at the diestrous stage, but did not reach a statistical significance (Fig.18A).

To further confirm that the increased luciferase activity at the proestrous stage was due to the activation of the hTFF1 promoter by the increased serum hormone during the natural estrous cycles, we measured the serum hormone levels from *hTFF1-luc*<sup>+/+</sup> rats at different estrous stages (table 5). We found that the highest serum estradiol level in the *hTFF1-luc*<sup>+/+</sup> transgenic rat was at the proestrous stage  $88.7 \pm 7.1$  pg/ml, with the lowest estradiol level at the diestrous stage  $34.8 \pm 3.2$  pg/ml. The serum progesterone level was highest at the diestrous stage with  $19.8 \pm 2.8$  ng/ml, and lowest at the estrous stage with  $7.7 \pm 1.1$  ng/ml. This was in accordance with the physiological serum estrogen and progesterone levels in the rats. We checked the correlation between the luciferase activity in the brain at the proestrous and estrous stages with the serum estradiol level at the proestrous and estrous stages. It was shown that the luciferase activity in the brain at the proestrous and estrous stages was highly correlated to the serum estradiol level at

the proestrous and estrous stages, with a correlation coefficient of 0.980 (Fig.18B). This indicated that the hTFF1 promoter was activated by estradiol and led to an increase of luciferase activity in the tissues.

**Table 5. Serum estradiol and progesterone levels from the *hTFF1-luc*<sup>+/+</sup> rats at different estrous stages**

<b>Estrous phase</b>	<b>Estradiol(pg/ml)</b>	<b>Progesterone(ng/ml)</b>
Estrous	34.9±2.6	10.9±1.5
Proestrous	88.7±7.1	7.7±1.1
Diestrous	34.8±3.2	19.8±2.8



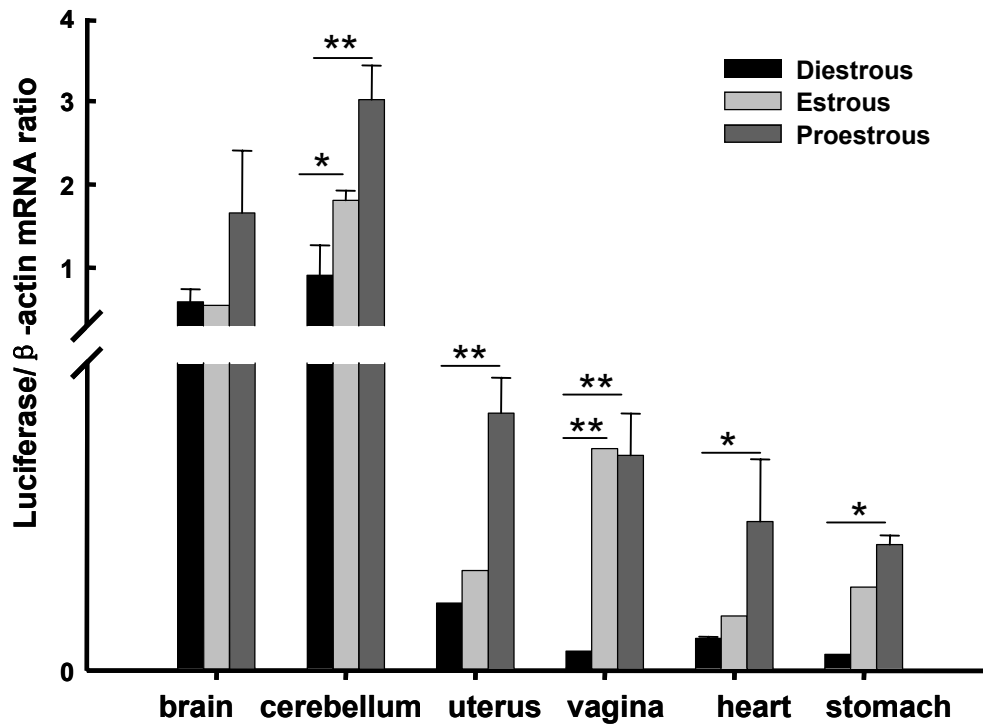
**FIG. 18. Identification of the activation of the hTFF1 promoter by the serum estradiol**

**A:** luciferase activity in the tissues from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at different estrous stages. The luciferase activity in brain from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at proestrous stage was significantly increased when compared to that at the diestrous stage. Each bar represented an average of luciferase activity from three individual *hTFF1-luc*<sup>+/+</sup> transgenic rats (n=3). Mann-Whitney-U-test was used for statistical analysis. \*\*indicates p<0.01 compared the luciferase activity in brain and cerebellum at proestrous with that at diestrous in *hTFF1-luc*<sup>+/+</sup> transgenic rats.

**B:** Correlation of luciferase activity in brain with the serum estradiol level from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at proestrous and estrous stages. The luciferase activity in brain from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at proestrous and estrous stages was highly correlated with the serum estradiol level (summarized in table 5) with a coefficient of 0.980 at the proestrous and estrous stages.

### **3. 2. 4. 3. Luciferase mRNA was upregulated in the tissues from *hTFF1-luc*<sup>+/+</sup> rats at proestrous.**

To investigate whether the hTFF1 promoter can be activated by serum estradiol and lead to an increase of luciferase at mRNA level during the natural estrous cycle, real-time PCR was performed to quantify the luciferase mRNA level in the tissues from *hTFF1-luc*<sup>+/+</sup> transgenic mice at different estrous stages. The real-time PCR revealed that the luciferase mRNA in brain ( $1.66 \pm 0.75$ ) and cerebellum ( $3.02 \pm 0.41$ ) at the proestrous stage was 2.8-fold and 3.3-fold higher, respectively, than that in the brain ( $0.59 \pm 0.16$ ) and cerebellum ( $0.91 \pm 0.36$ ) at the diestrous stage (Fig. 19), respectively. This result was consistent with the result obtained by the luciferase activity assay in section of 3. 2. 4. 3. The luciferase mRNA level at the proestrous stage in heart, stomach, uterus and vagina was significantly increased by 4-, 7-, 3.8-, and 11-fold, respectively, when compared to the luciferase activity in the corresponding tissue from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at diestrous stage (Fig.19).



**FIG. 19. Real-time quantitative PCR analysis of luciferase mRNA in different tissues from female *hTFF1-luc*<sup>+/+</sup> transgenic rats at different estrous stages**

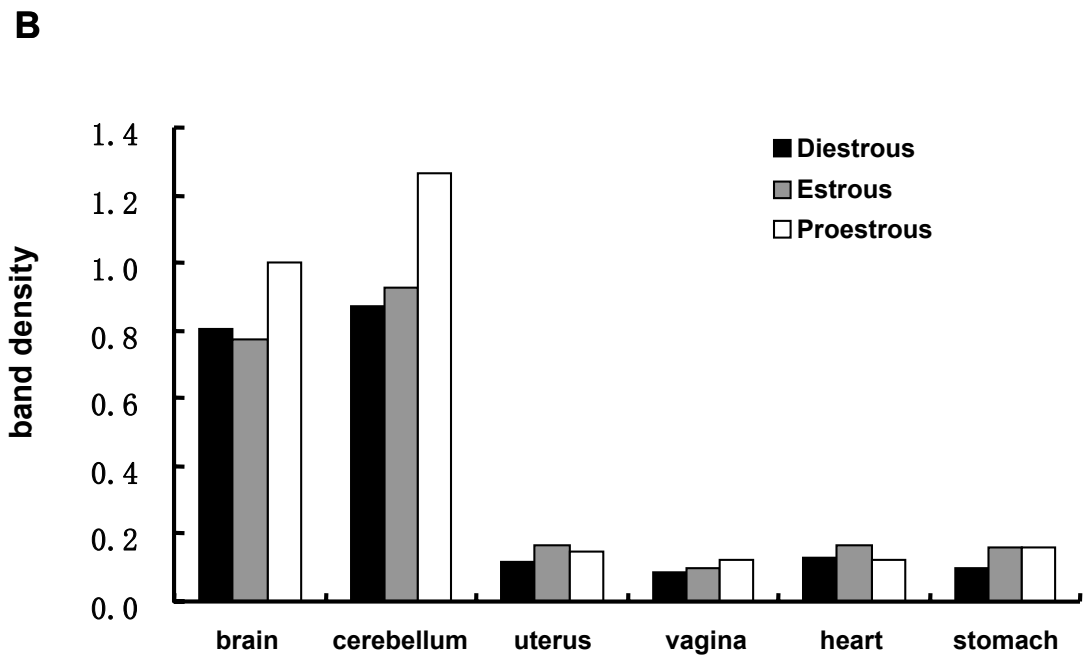
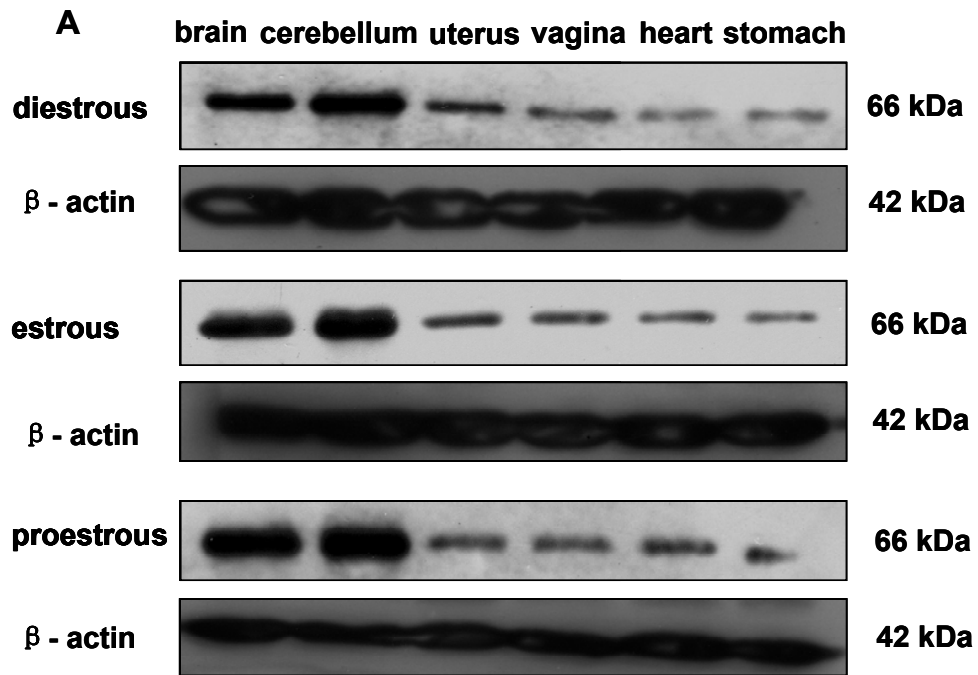
Real-time quantitative PCR analysis revealed that the luciferase transcription was enhanced at proestrous stage when compared to the diestrous stage. Total RNA was isolated from the tissues of female *hTFF1-luc*<sup>+/+</sup> transgenic rats at different estrous stages and purified. Real-time PCR quantification of luciferase was performed as described in the materials and methods section, with the use of luciferase-specific primers and taqman probe. Every sample was performed in triplicate. The level of luciferase mRNA gene was normalized to a housekeeping gene  $\beta$ -actin. Data were expressed as the ratio of luciferase mRNA to  $\beta$ -actin mRNA. n=3, T-test was used for statistical analysis. \*p<0.05, \*\*p<0.01 compared the luciferase mRNA in the tissues from the *hTFF1-luc*<sup>+/+</sup> rats at proestrous stage with that at diestrous stage.

### **3. 2. 4. 4. Luciferase protein was upregulated in the tissues from the *hTFF1-luc*<sup>+/+</sup> rats at the proestrous stage.**

To address the conflicting results of the luciferase activity and mRNA level in the uterus, vagina, heart and stomach, western blot was employed to examine the luciferase expression in the tissues from *hTFF1-luc*<sup>+/+</sup> rats at different estrous stages. Western blot



analysis of the luciferase protein revealed that there were no differences in the luciferase expression between the tissues of heart, stomach, uterus and vagina from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at the same estrous stage (Fig. 20 A and B). And the western blot analysis also showed that there were no differences in the luciferase expression in the same tissues of heart, stomach, uterus and vagina from the *hTFF1-luc*<sup>+/+</sup> transgenic rats at different estrous stages (Fig. 20 A and B). However, at the proestrous stage, the luciferase protein expression in the brain and cerebellum was significantly increased when compared to that at the estrous and diestrous stages (Fig. 20 A and B).



**FIG. 20. Representative western blot analysis of luciferase protein expression in different tissues from female *hTFF1-luc*<sup>+/+</sup> transgenic rats at different estrous stages**

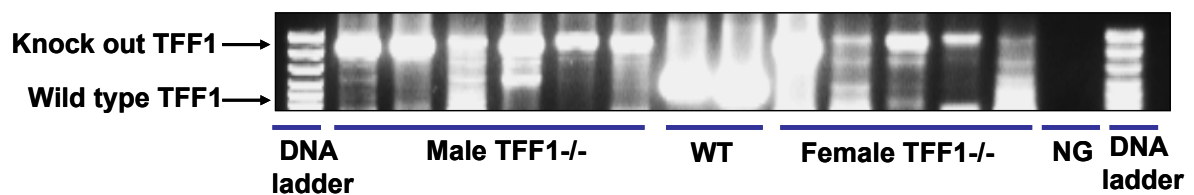
**A:** representative immunoblots are shown together with corresponding immunoblot for actin.

**B:** corresponding graphs depict densitometric analysis of luciferase band in relation to the corresponding actin band. Data were normalized to the densitometry of the luciferase band at proestrous in brain, which was depicted as 1.0 in the column of the graph. There were no differences in the luciferase expression between the tissues of heart, stomach, uterus and vagina in the *hTFF1-luc<sup>+/+</sup>* transgenic rats at the same estrous stage. And there were no differences in the luciferase expression in the same tissues of heart, stomach, uterus and vagina in the *hTFF1-luc<sup>+/+</sup>* transgenic rats at different estrous stages. However, at the proestrous stage, the luciferase expression in the brain and cerebellum was significantly increased when compared to that at the estrous and diestrous stages.

### 3. 3. *In vivo* experiments II

#### 3. 3.1.Characterization of the TFF1 knock out mice

The TFF1 knock out mice were generated by targeted disruption of the mouse TFF1 gene in the exon 2, which contains P domain sequence, using a pRH1-4 neo targeting construct. No TFF1 mRNA and protein were detected in the TFF1 knock out mice (Lefebvre et al., 1996). We further identified the disrupted TFF1 gene by PCR. It was shown that in the TFF1 knock out mice, the length of disrupted TFF1 PCR product was larger than that of wild type mice (Fig. 21). This is because a targeting construct was inserted into the exon 2 of the TFF1 to inactivate the TFF1 gene.



**FIG. 21. Identification of the TFF1 knock out mice by PCR**

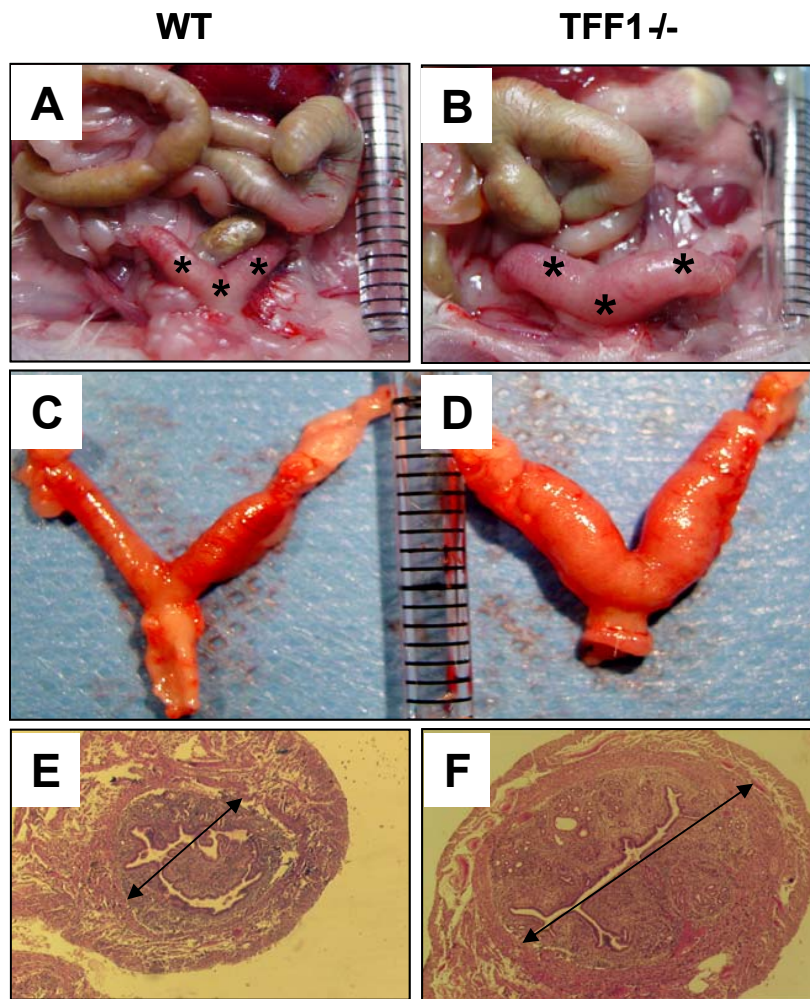
Genomic DNA was isolated from the wild type (129 Svj) and TFF1 knock out mice. TFF1 knock out mice were identified with a larger PCR product in length when compared to that from the wild type mice.

### **3. 3. 2. Morphological alteration of uterus and vagina in the female TFF1 knock out mice**

#### **3. 3. 2. 1. Loss of TFF1 led to uterine hyperplasia**

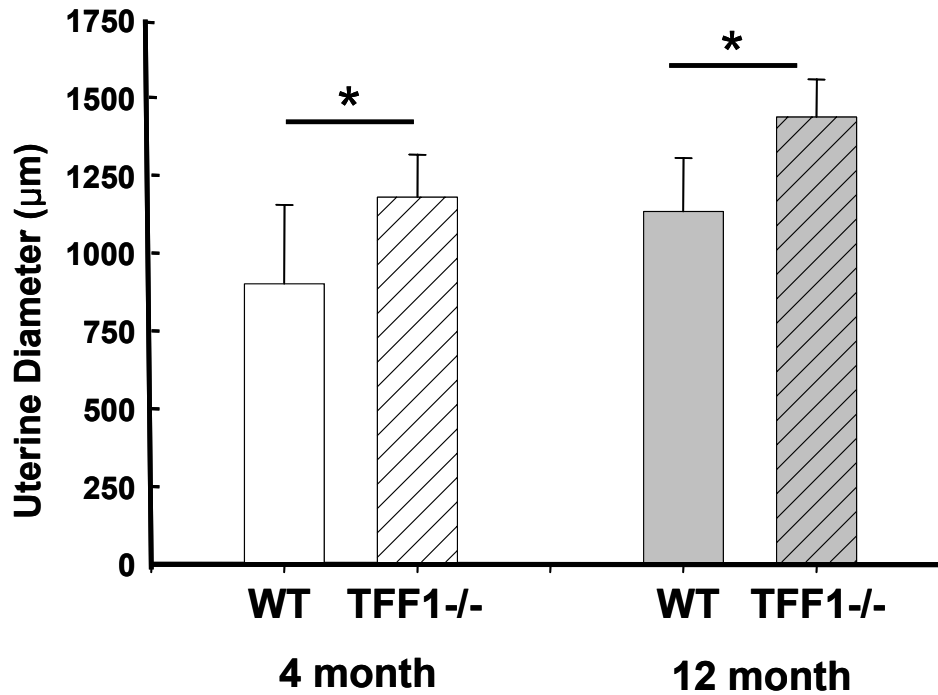
To examine the impact of loss of TFF1 on the uterine and vaginal morphology, TFF1 knock out 12-month-old female mice at estrous and diestrous stages were sacrificed for morphological analysis. Striking morphological alterations were observed in TFF1 knock out mice. The uterus from TFF1 knock out mice was much bigger than that of wild type mice at the same estrous stage (Fig. 22), and had a much higher ratio of relative uterine weight (uterus to body weight) (Fig. 24). It was shown that the relative uterine weight of TFF1 knock out mice ( $0.0220 \pm 0.000168$ ) was almost two-fold that of wild type mice ( $0.0113 \pm 0.000129$ ) (Fig. 24), suggesting that loss of TFF1 led to the uterine hyperplasia. However, the uterine alteration was not observed in the 5-month-old TFF1 knock out mice (Lefebvre et al., 1996).

Next, we measured and compared the uterine diameter between the wild type and TFF1 knock out mice. It was shown that the uterine diameter from TFF1 knock out mice was significantly increased when compared to the wild type mice. The uterine diameter of 12-month-old TFF1 knock out mice was  $1436.8 \pm 121.8 \mu\text{m}$ , whereas the uterine diameter of wild type mice of the same age was  $1133.6 \pm 172.1 \mu\text{m}$ . The same result was observed in 4-month-old TFF1 knock out mice. The uterine diameter of 4-month-old wild type mice was  $900.8 \pm 254.1 \mu\text{m}$ , whereas the uterine diameter of TFF1 knock out mice of the same age was  $1180.0 \pm 136.2 \mu\text{m}$ .



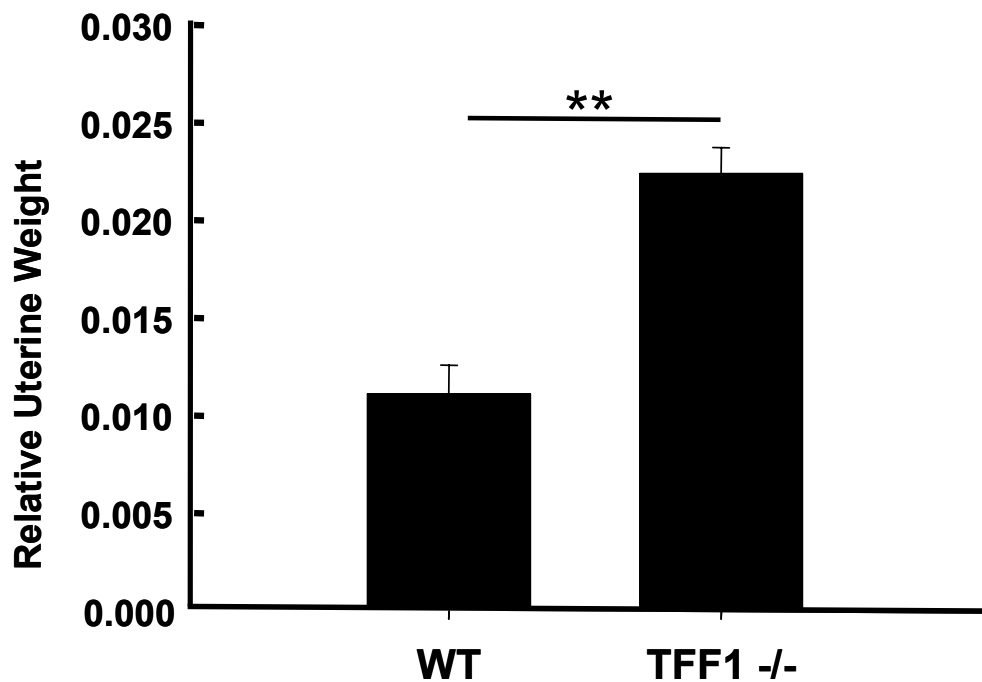
**FIG. 22. Morphological analysis of representative uterus from 12-month-old TFF1 knock out and wild type mice at estrous stage**

The 12-month-old wild type and TFF1 knock out mice at estrous stage were sacrificed and the tissues were harvested for further experiment. **ABCD**: the appearance of uterus from 12-month-old wild type and TFF1 knock out mice at estrous stage. It was shown that the uterus from 12-month-old, but not 4-month-old TFF1 knock out mice, was much bigger with a two-fold increased ratio of relative uterine weight than that of wild type mice. **EF**: uterine diameter of TFF1 knock out mice at estrous stage was significantly increased when compared to that of wild type mice, original magnification  $\times 25$ .  $n=4-6$ .



**FIG. 23. Statistical analysis of the uterine diameter of 4-month- and 12-month-old wild type and TFF1 knock out mice at estrous stage**

The uterine diameter of 4-month- and 12-month-old TFF1 knock out mice at estrous stage was significantly increased when compared to that of wild type mice at the same age. The measurement of uterine diameter was based on the analysis of three sections for each uterine specimen. Quantification was performed on the digitized images of three systematic, randomly selected, representative fields and was reported as the mean±SD. The uterine diameter of 4-month-old wild type mice was 900.8±254.1 µm, whereas the uterine diameter of 4-month-old TFF1 knock out mice was 1180.0±136.2 µm. The uterine diameter of 12-month-old wild type mice was 1133.6±172.1 µm, whereas the uterine diameter of 12-month-old TFF1 knock out mice 1436.8±121.8 µm. U-test was used for statistical analysis. n=4-6, \* represents P<0.05.



**FIG. 24. Statistical analysis of the ratio of relative uterine weight of 12-month-old wild type and TFF1 knock out mice at estrous stage**

The relative uterine weight of wild type mice was  $0.0113 \pm 0.000129$ , whereas the relative uterine weight of TFF1 knock out mice was  $0.0220 \pm 0.000168$ , which was almost two times greater than that of wild type mice. 12-month-old wild type and TFF1 knock out mice were sacrificed and the uterus was harvested and weighed. The ratio of relative uterine weight (the uterine weight to the body weight) of TFF1 knock out mice was significantly increased when compared to that of wild type mice. U-test was used for statistical analysis.  $n=4-6$ , \*\* represents  $P < 0.01$ .

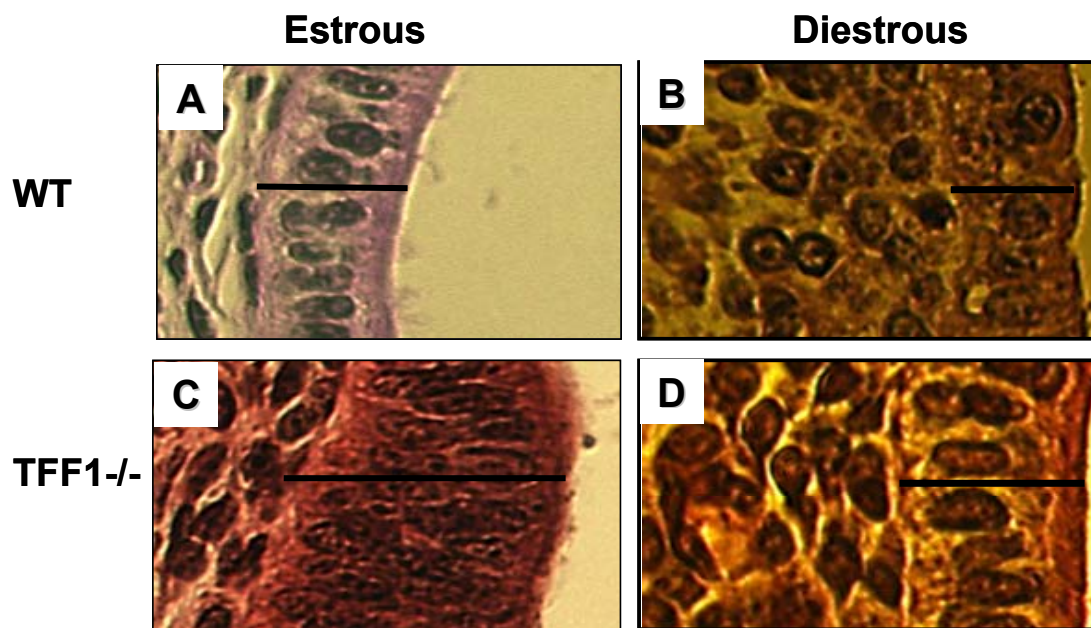
### **3. 3. 2. 2. Loss of TFF1 led to increased thickness of total uterine and vaginal epithelial layer.**

To further investigate the uterine hyperplasia and the uterine and vaginal histological alteration of 12-month-old TFF1 knock out mice, we further examined the histology of uterus and vagina from TFF1 knock out mice. Significantly increased epithelial height or thickness of the total uterine epithelium was observed in TFF1 knock out mice at estrous and diestrous stages, when compared to wild type mice (Fig. 25; Fig. 26). The uterine

epithelial height of wild type mice at estrous stage was  $19.2\pm 6.6\ \mu\text{m}$ , whereas the uterine epithelial height of TFF1 knock out mice at estrous stage was  $25.7\pm 6.9\ \mu\text{m}$ . The uterine epithelial height of wild type mice at diestrous was  $16.4\pm 3.9\ \mu\text{m}$ , whereas the uterine epithelial height of TFF1 knock out mice at diestrous stage was  $22.5\pm 6.3\ \mu\text{m}$ . No alteration in the stratification of the uterine epithelium was observed in the TFF1 knock out mice at estrous or diestrous stages.

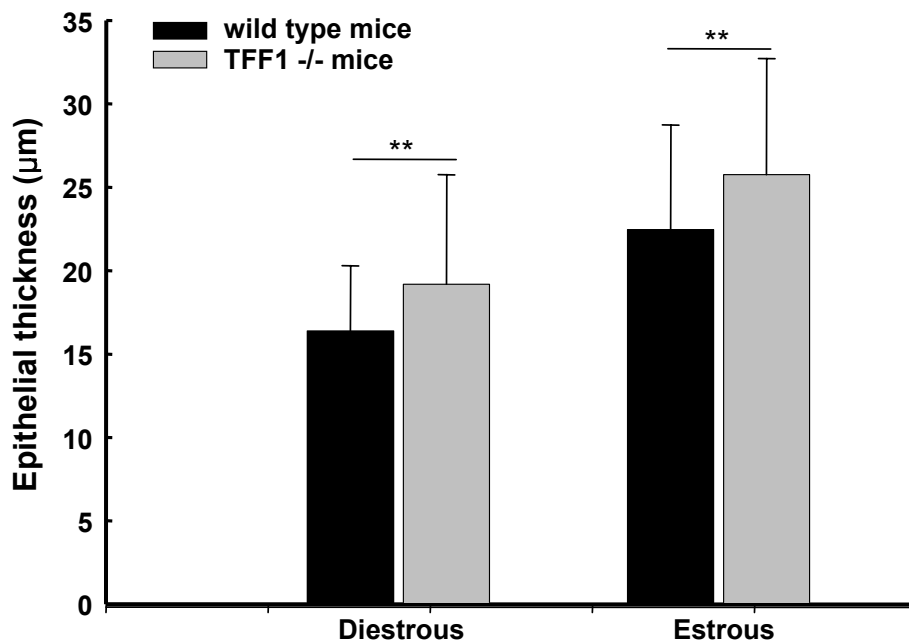
The same alteration was observed in the vagina from TFF1 knock out mice as well. The pseudostratified hyperplastic epithelium was observed in TFF1 knockout mice. It was shown that the vaginal epithelium from the wild type mice at estrous stage was formed by 6–8 layers, of which superficial layers were cornified, with a total vaginal epithelial thickness of  $111.5\pm 39.5\ \mu\text{m}$ , whereas the vaginal epithelium from the TFF1 knock out mice at estrous was formed by more than 10 layers, of which superficial layers were cornified as well, with a total vaginal epithelial thickness of  $140.1\pm 25.6\ \mu\text{m}$  (Fig 27; Fig. 28). At diestrous stage, the vaginal epithelial thickness in the wild type and TFF1 knock out mice was reduced in epithelial layers when compared to the mice at estrous stage. However, the total vaginal epithelial thickness from TFF1 knock out mice at diestrous stage was  $101.9\pm 46.6\ \mu\text{m}$ , which was still significantly higher than that of wild type mice at diestrous stage with a total epithelial thickness of  $64.2\pm 26.8\ \mu\text{m}$  (Fig. 27; Fig. 28).





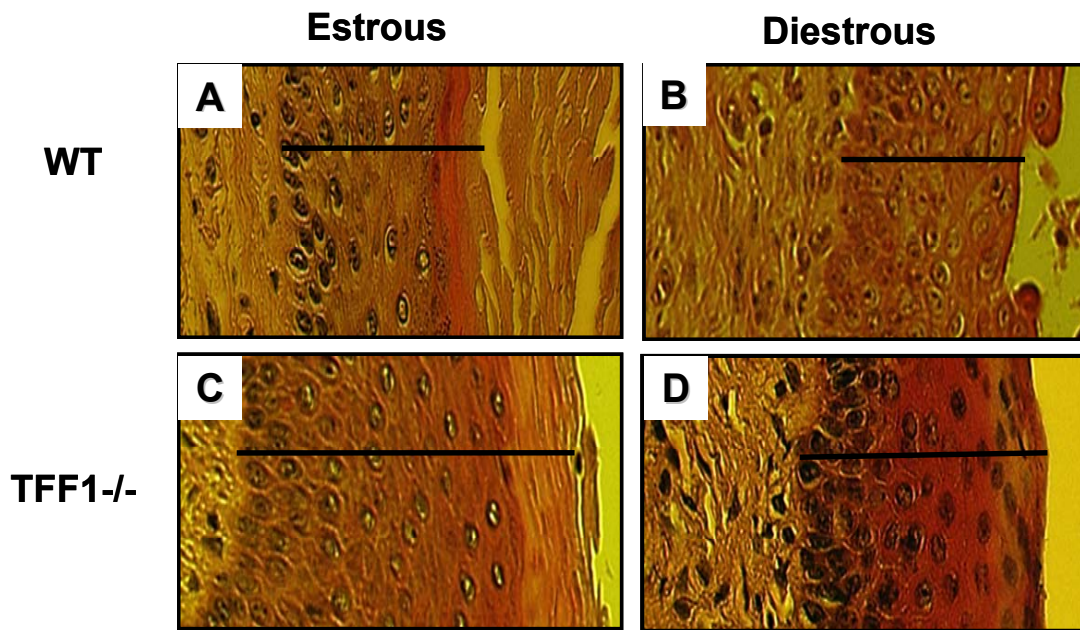
**FIG. 25. Histological analysis of representative uterine epithelial height or total thickness from wild type and TFF1 knock out mice at estrous and diestrous stages**

The height or thickness of total uterine epithelium cells was significantly increased in TFF1 knock out mice at estrous and diestrous stages (**C** and **D**) when compared to wild type mice (**A** and **B**).The histopathological alterations were examined under a light microscope. n=4-6.



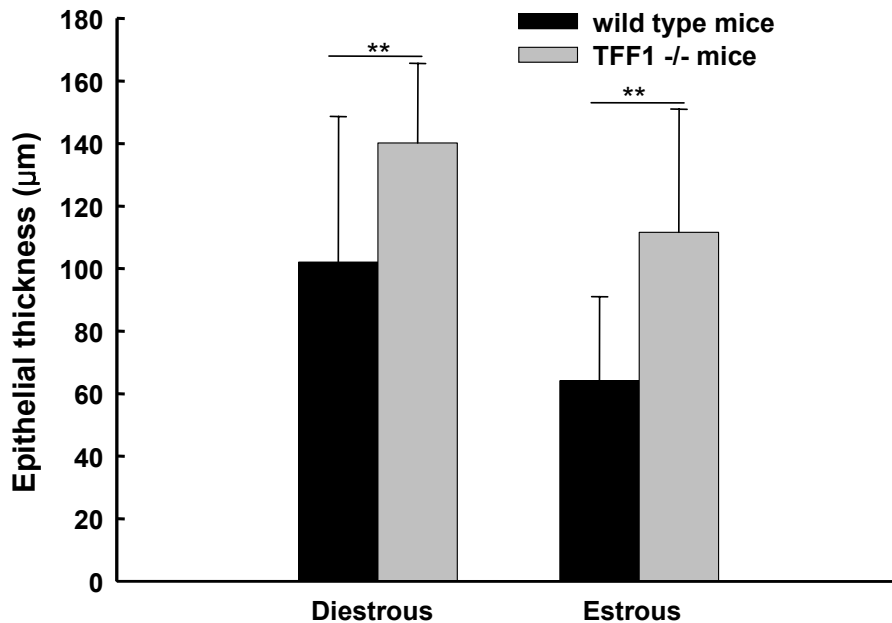
**FIG. 26. Statistical analysis of the thickness of representative uterine epithelium from wild type and TFF1 knock out mice at estrous and diestrous stages**

Significantly increased uterine epithelial height or the thickness of total epithelium was observed in TFF1 knock out mice at estrous and diestrous stages. The histopathological alterations were examined under a light microscope. The thickness of uterine epithelial layers was measured using a light microscope and the public domain Scion Image Analysis software. The measurement of uterine epithelial thickness was based on the analysis of three sections for each uterine specimen. Quantification was performed on the digitized images of 9 systematic, randomly selected, representative fields and was reported as the mean±SD. The uterine epithelial height or epithelial thickness of wild type mice at estrous stage was 19.2±6.6 µm, whereas the uterine epithelial height of TFF1 knock out mice at estrous stage was 25.7±6.9 µm. The uterine epithelial height in wild type mice at diestrous was 16.4±3.9 µm, whereas the uterine epithelial height of TFF1 knock out mice at diestrous was 22.5±6.3 µm. Original magnification×400. U-test was used for statistical analysis. n=4-6, \*\* represents P<0.01.



**FIG. 27. Histological analysis of representative total thickness of vaginal epithelium from wild type and TFF1 knock out mice at estrous and diestrous stages**

Pseudostratified hyperplastic vaginal epithelium was observed in TFF1 knockout mice. The vaginal epithelium in the TFF1 knock out mice at the estrous stage was formed by more than 10 layers (**C**), whereas the vaginal epithelium in wild type mice at the estrous stage was formed by 6–8 layers, of which superficial layers were cornified (**A**). The height or thickness of total vaginal epithelium cells was significantly greater in TFF1 knock out mice at estrous and diestrous stages (**C** and **D**) than that of wild type mice (**A** and **B**). n=4-6.



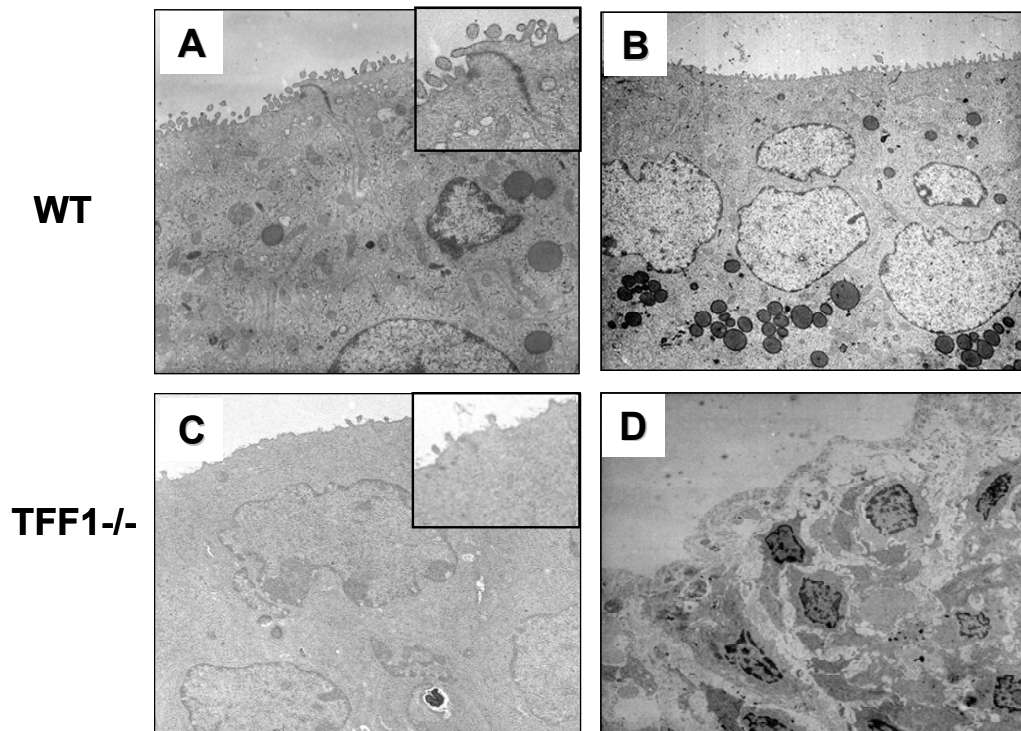
**FIG. 28. Statistical analysis of the total thickness of representative vaginal epithelium from wild type and TFF1 knock out mice at estrous and diestrous stages**

Significantly increased epithelial thickness of the total vaginal epithelium was observed in TFF1 knock out mice at estrous and diestrous stages. The histopathological alterations were examined under a light microscope. The thickness of vaginal epithelial layers was measured using a light microscope and the public domain Scion Image Analysis software. The measurement of vaginal epithelial thickness was based on the analysis of three sections for each vaginal specimen. Quantification was performed on the digitized images of 9 systematic, randomly selected, representative fields and was reported as the mean $\pm$ SD. The vaginal epithelial height or epithelial thickness from wild type mice at estrous stage was 111.5 $\pm$ 39.5  $\mu$ m, whereas the vaginal epithelial height from TFF1 knock out mice at estrous stage was 140.1 $\pm$ 25.6  $\mu$ m. The vaginal epithelial height in wild type mice at diestrous stage was 64.2 $\pm$ 26.8  $\mu$ m, whereas the uterine epithelial height of TFF1 knock out mice at diestrous stage was 101.9 $\pm$ 46.6  $\mu$ m. Original magnification $\times$ 400. U-test was used for statistical analysis. n=4-6, \*\* represents P<0.01.

### **3. 3. 2. 3. Loss of TFF1 led to loss of cell-cell contact and cell-cell adhesion in the uterine and vaginal epithelium.**

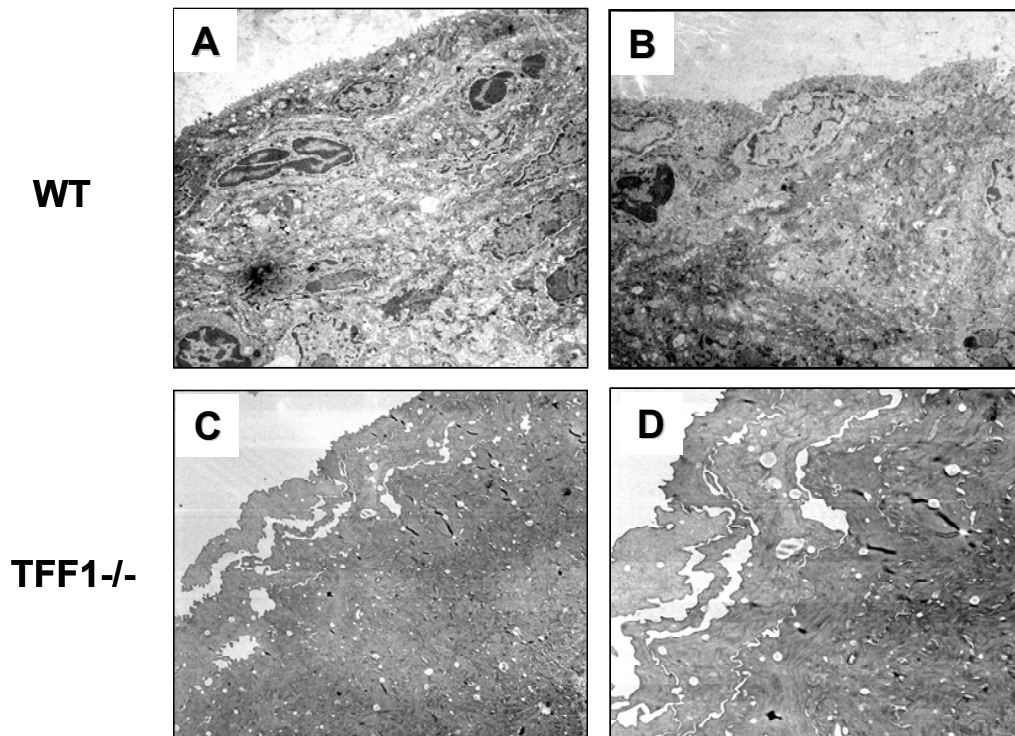
To further investigate the uterine alterations in its microstructure, electron microscopy analysis was employed. Electron microscopy analysis of the uterus from the TFF1 knock out mice revealed that epithelial cells lost the cell-cell contact and appeared to lose the cell adhesion with the neighboring epithelial cells (Fig. 29), which, in turn, might trigger the increased uterine epithelial height in the TFF1 knock out mice. Furthermore, the epithelial cells lost almost all the microvilli on their surface. These alterations were observed even in 2-month-old TFF1 knock out mice. In contrast, the epithelial cells in the uterus from wild type mice exhibited perfect cell-cell contact with the neighboring cells and had a clear cell structure with a lot of microvilli in a good condition on their surface (Fig.29).

These alterations were also observed in the vagina from TFF1 knock out mice. It was shown that the vaginal epithelial cells lost the cell-cell contact, which was observed even in 2-month-old TFF1 knock out mice. Some of the epithelial cells on the top layer of the vagina were almost completely damaged and degraded. The epithelial cells lost almost all the microvilli on their surface (Fig. 30). In contrast, the vaginal epithelial cells of wild type mice exhibited perfect cell-cell contact with the neighboring cells and had a clear cell structure with a lot of microvilli on their surface (Fig. 30).



**FIG. 29. Electron microscopy analysis of the microstructure alterations in the uterus from the TFF1 knock out mice**

Electron microscopy analysis revealed that the uterine epithelial cells lost the cell-cell contact and cell-cell adhesion with neighboring epithelial cells, which was observed even in 2-month-old TFF1 knock out mice. Furthermore, the uterine epithelial cells of TFF1 knock out mice lost almost all the microvilli on their surface (C and D). In contrast, the uterine epithelial cells of wild type mice exhibited perfect cell-cell contact and had a clear cell structure with a lot of microvilli on their surface (A and B). n=3.



**FIG. 30. Electron microscopy analysis of the microstructure alterations in the vagina from the TFF1 knock out mice**

In TFF1 knock out mice, the vaginal epithelial cells lost the cell-cell contact and appeared to lose the cell adhesion with the neighboring epithelial cells. Some of the epithelial cells on the top layer of the vagina were almost completely damaged and degraded. And the epithelial cells lost almost all the microvilli on their surface (C and D). In contrast, the vaginal epithelial cells of wild type mice exhibited perfect cell-cell contact and had a clear cell structure with a lot of microvilli on their surface (A and B). n=3.

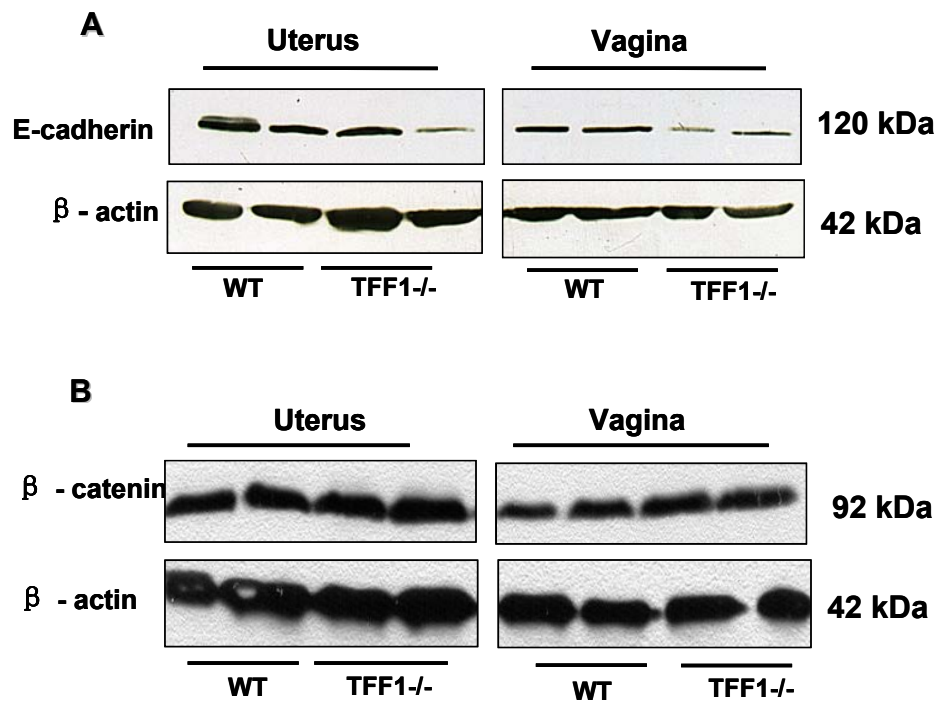
### **3. 3. 2. 4. Loss of TFF1 led to downregulation of E-cadherin but not $\beta$ -catenin expression in the uterus and vagina.**

Loss of cell-cell contact was distinctly visible by electron microscopy analysis in the uterine and vaginal epithelial cells in TFF1 knock out mice. E-cadherin, a transmembrane glycoprotein, mediates calcium-dependent intercellular adhesion and is specifically involved in the epithelial cell-to-cell adhesion (Gumbiner, 2000). Together with  $\beta$ -catenin, E-cadherin plays an important role in the adhesion junction of the epithelial cells in the

vertebrate (Knust, 2002). In addition, *in vitro* promoter assay, we have already shown that in the presence of ER  $\alpha$ , TFF1 was able to activate the E-cadherin promoter and upregulate its expression. Thus, we further studied the impact of the loss of TFF1 on the expression of E-cadherin and  $\beta$ -catenin in the uterus and vagina from the TFF1 knock out mice using western blot. It was shown that E-cadherin expression in the uterus and vagina from TFF1 knock out mice was significantly decreased when compared to the wild type mice (Fig. 31A), whereas no significant difference in  $\beta$ -catenin expression in uterus and vagina was observed between wild type and TFF1 knock out mice (Fig. 31B).

To further investigate whether the downregulated E-cadherin expression in the uterus and vagina from the TFF1 knock out mice occurred at transcriptional level, real-time quantitative PCR was performed to quantify E-cadherin mRNA as described in the materials and methods section. It was shown that the E-cadherin mRNA level in the uterus from TFF1 knock out mice was significantly downregulated when compared to the wild type mice (Fig. 32A). The E-cadherin mRNA level in the uterus from TFF1 knock out mice was  $17.8 \pm 16.6$ , whereas the E-cadherin mRNA level in the uterus from wild type mice was  $3.47 \pm 2.87$ . The downregulation of E-cadherin was also observed in the vagina of TFF1 knock out mice (Fig. 32B). It was shown that the E-cadherin mRNA level in the vagina from TFF1 knock out mice was  $0.53 \pm 0.47$ , whereas the E-cadherin mRNA level in the vagina from wild type mice was  $0.34 \pm 0.19$ . The downregulation of E-cadherin in the uterus and vagina from TFF1 knock out mice was confirmed by electrophoretic analysis of the aliquots of real-time PCR products on 2% agarose gel and visualized by staining with ethidium bromide (Fig. 32C). This alteration in E-cadherin mRNA level was even observed in 4-month-old TFF1 knock out mice.

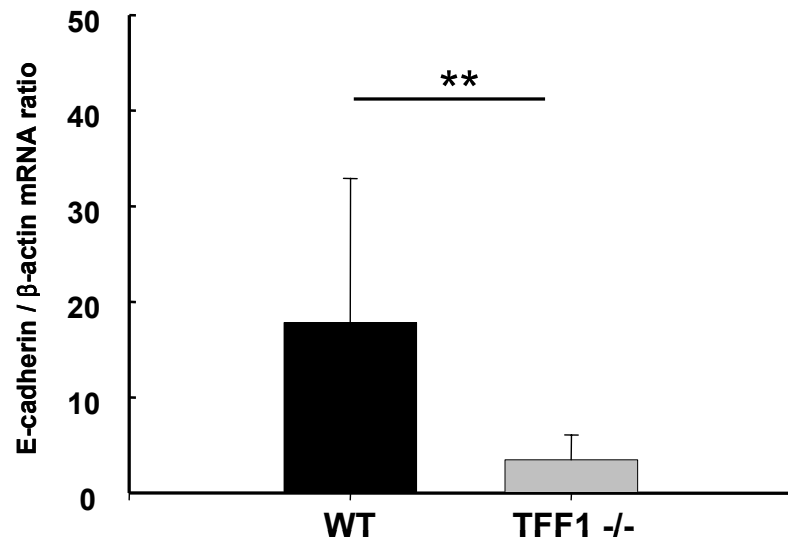




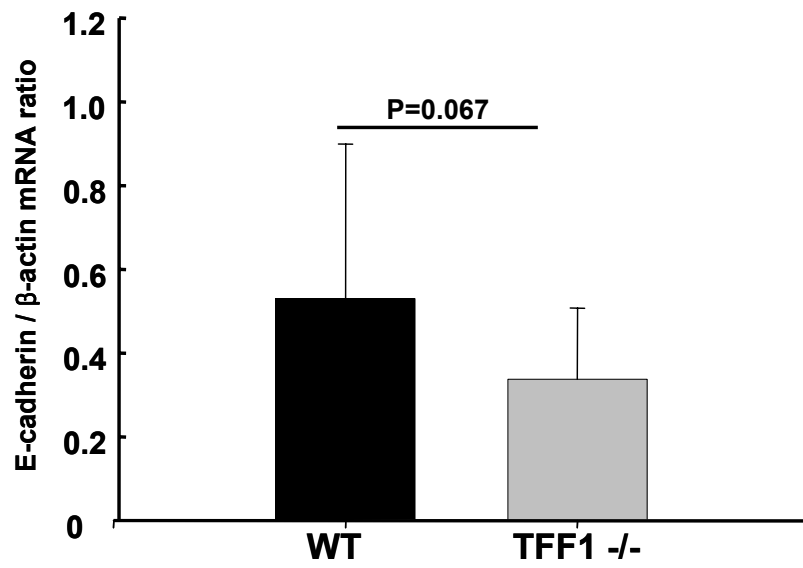
**FIG. 31. Representative western blot analysis of E-cadherin and  $\beta$ -catenin expression in the uterus and vagina from the wild type and TFF1 knock out mice**

Downregulation of E-cadherin expression of in the uterus and vagina from TFF1 knock out mice was observed when compared to the wild type mice. However, no difference in  $\beta$ -catenin expression was observed in the uterus and vagina between TFF1 knock out mice and wild type mice. E-cadherin (120 KDa) and  $\beta$ -catenin (92 KDa) were normalized to the housekeeping gene  $\beta$ -actin at 42 kDa, respectively. n=4.

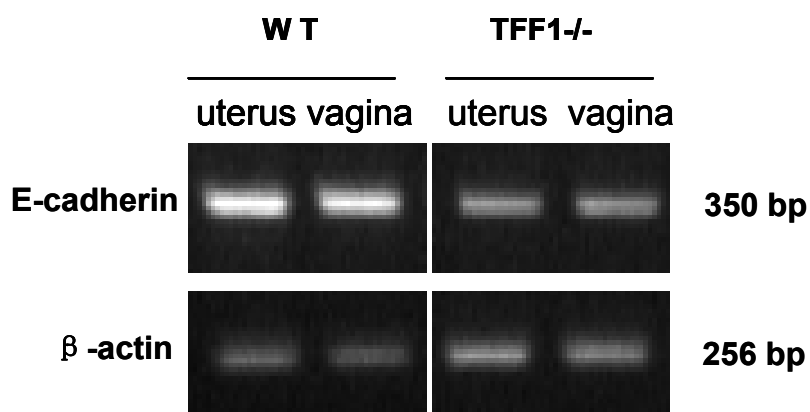
**A**



**B**



**C**



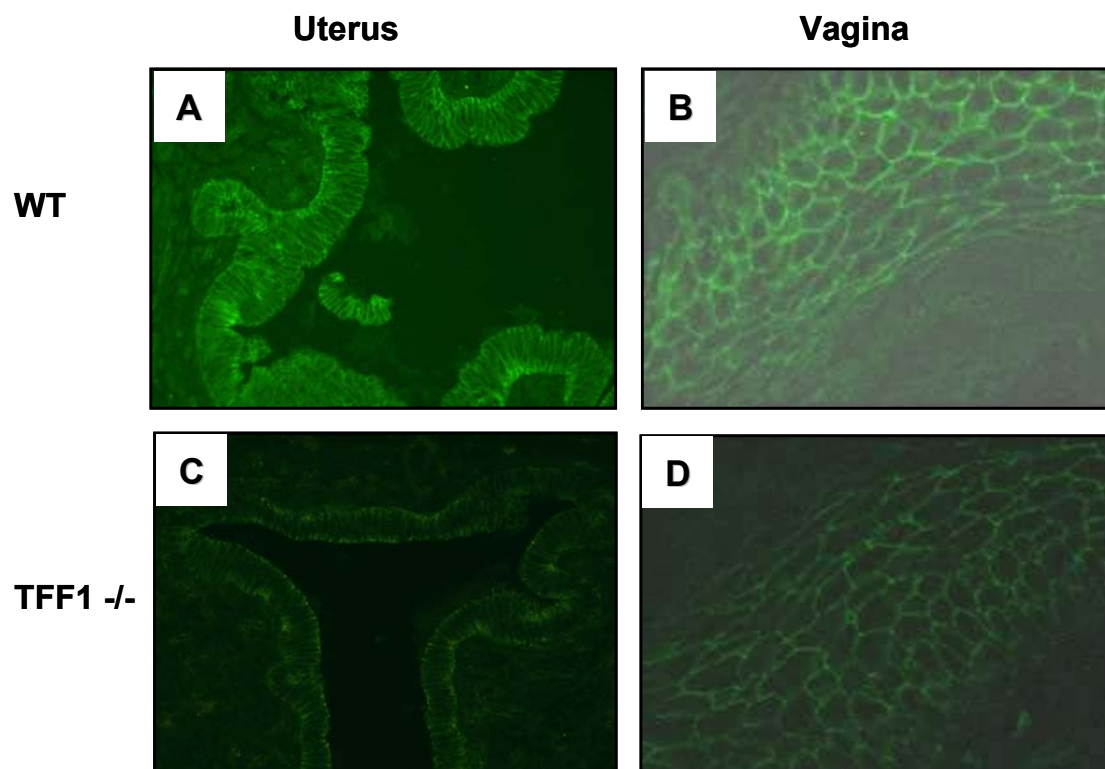
**FIG. 32. Real-time quantitative PCR analysis of E-cadherin mRNA level in uterus and vagina from wild type and TFF1 knock out mice**

Real-time PCR was performed in triplicate for each sample. E-cadherin mRNA was normalized to a housekeeping gene  $\beta$ -actin. It was shown that E-cadherin was downregulated at the mRNA level in the uterus **(A)** and vagina **(B)** from TFF1 knock out mice when compared to that of wild type mice. Data were expressed as the ratio of E-cadherin mRNA to  $\beta$ -actin mRNA. E-cadherin mRNA level in the uterus from TFF1 knock out mice was  $17.8 \pm 16.6$ , whereas in the uterus from wild type mice it was  $3.47 \pm 2.87$ ; E-cadherin mRNA level in the vagina from TFF1 knock out mice was  $0.53 \pm 0.47$ , whereas in the vagina from wild type mice it was  $0.34 \pm 0.19$ . Aliquots of real-time PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by staining with ethidium bromide to exclude the non-specific PCR binding **(C)**. U-test was used for statistical analysis.  $n=3$ ,  $**p < 0.01$  compared the E-cadherin mRNA in uterus and vagina of wild type mice.

### **3. 3. 2. 5. Loss of TFF1 was unable to trigger the translocation of E-cadherin and $\beta$ -catenin from epithelial cell membrane to cytoplasm or nucleus.**

To further confirm the downregulation of E-cadherin in uterus and vagina from TFF1 knock out mice obtained by western blot, and to investigate whether loss of TFF1 might trigger the translocation of E-cadherin and  $\beta$ -catenin from epithelial cell surface membrane to cytoplasm or nucleus, immunofluorescence analysis was employed. The immunofluorescence analysis of E-cadherin and  $\beta$ -catenin revealed that the uterine and

vaginal epithelial cells consistently demonstrated a typical pattern of E-cadherin and  $\beta$ -catenin expression at the cell borders, with unequivocal and strong membranous staining localized at the cell surface membrane, primarily at cell-cell adhesion sites in TFF1 knock out and wild type mice. However the staining intensity of the immunofluorescence of E-cadherin in the uterine and vaginal epithelial cells from TFF1 knock out mice was significantly decreased compared to that of the wild type mice (Fig. 33).

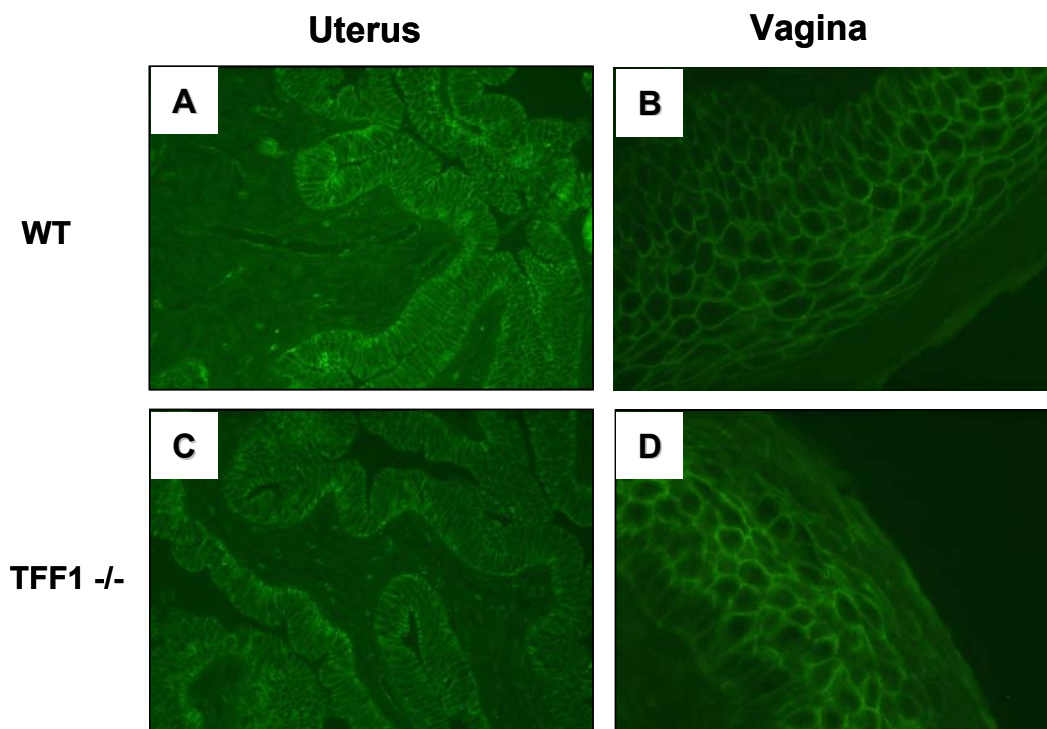


**FIG. 33. Immunofluorescence analysis of the localization and expression of E-cadherin in uterus and vagina from wild type and TFF1 knock out mice**

Immunofluorescence analysis revealed that the uterine and vaginal epithelial cells consistently exhibited a typical pattern of E-cadherin expression at the cell borders, with unequivocal and strong membranous staining localized at the cell surface membrane, primarily at cell-cell adhesion sites in wild type and TFF1 knock out mice. However the staining intensity of the immunofluorescence of E-cadherin in the uterine and vaginal epithelial cells from TFF1 knock out mice (**C** and **D**) was

significantly decreased compared to that of the wild type mice (**A** and **B**). No localization alteration of E-cadherin was observed in the uterine or vaginal epithelial cells from TFF1 knock out mice. n=3.

This alteration was observed even in 2-month-old TFF1 knock out mice, suggesting that loss of TFF1 led to downregulation of E-cadherin expression in uterus and vagina. This was consistent with the result obtained by western blot and real-time PCR. However, no translocation of E-cadherin to the cytoplasm and nucleus was observed in the uterine or vaginal epithelial cells in TFF1 knock out mice. Also, no significant difference in staining intensity and localization of  $\beta$ -catenin was observed in the uterine or vaginal epithelial cells of TFF1 knock out mice when compared to the wild type mice (Fig. 34).



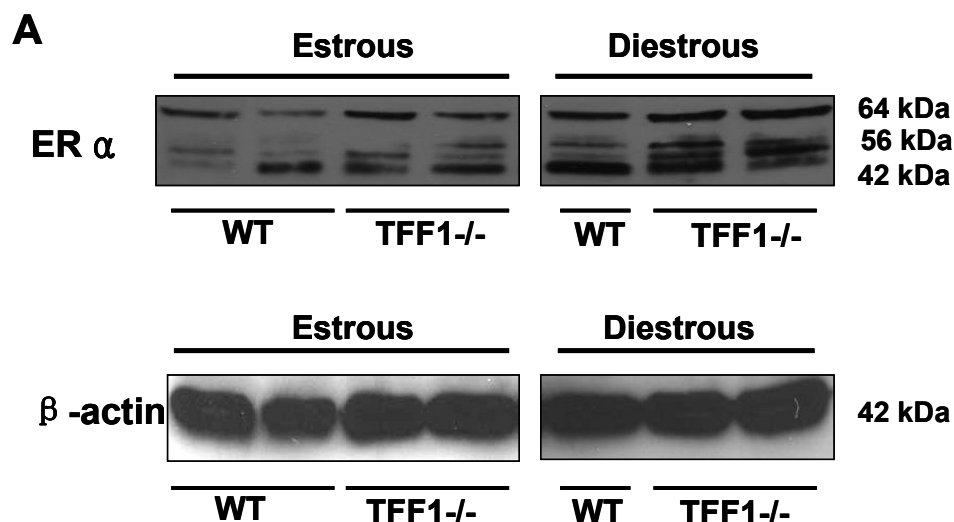
**FIG. 34. Immunofluorescence analysis of the localization and expression of  $\beta$ -catenin in uterus and vagina from wild type and TFF1 knock out mice**

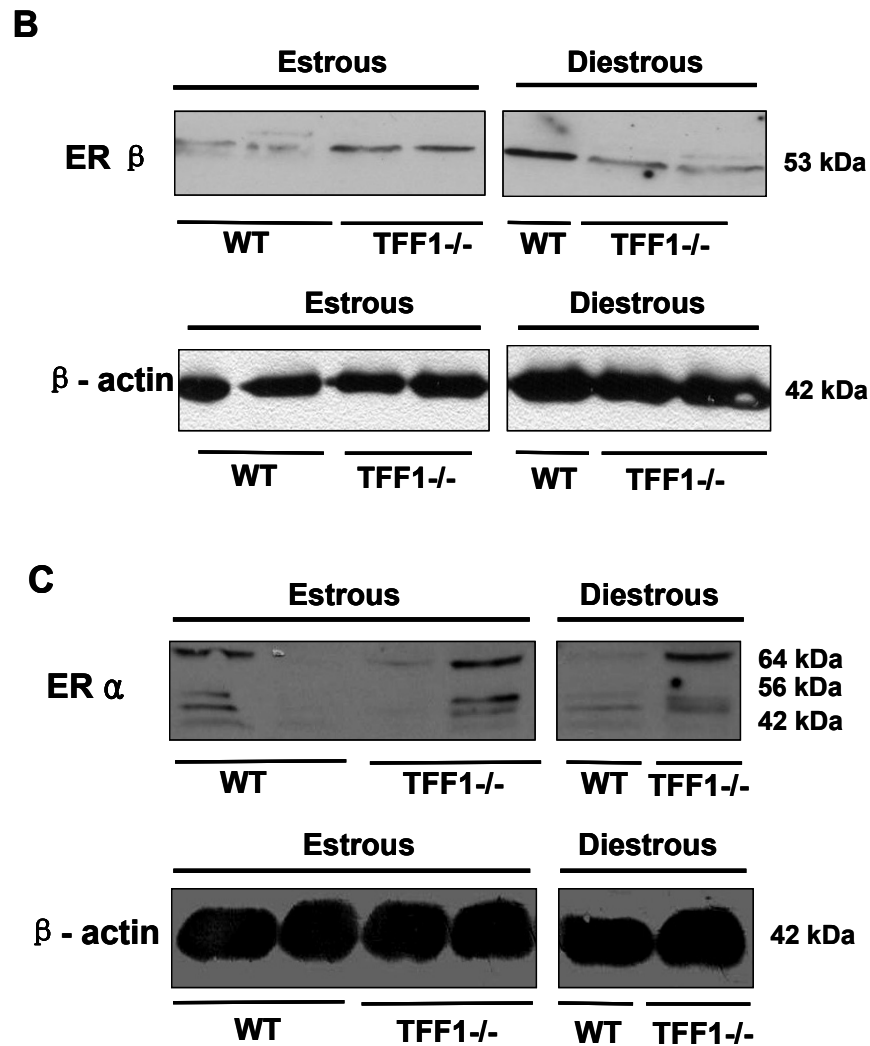
Immunofluorescence analysis revealed that the uterine and vaginal epithelial cells consistently exhibited a typical pattern of  $\beta$ -catenin expression at the cell borders, with unequivocal and strong membranous staining localized at the cell surface membrane, primarily at cell-cell adhesion sites in TFF1 knock out and wild type mice. However, no significant difference in staining intensity or

localization of  $\beta$ -catenin was observed in the uterine and vaginal epithelial cells from TFF1 knock out mice (**C** and **D**) when compared to the wild type mice (**A** and **B**). n=3.

### 3. 3. 2. 6. Loss of TFF1 led to increased expression of ER $\alpha$ but not ER $\beta$ in the uterus.

Since ER $\alpha$  plays an important role in the uterine and vaginal epithelial growth, morphogenesis, and cytodifferentiation (Gray et al., 2001; Galand et al., 1971), we investigated the ER $\alpha$  expression in the hyperplastic uterus and the vagina from the TFF1 knock out mice. Western blot analysis revealed that three bands of ER $\alpha$  were observed in the uterus at 64, 56, and 42 kDa, which are derived from the alternative usage of initiation ATG or splicing of ER $\alpha$  (Friend et al., 1995; Friend et al., 1997). The ER $\alpha$  expression was slightly increased at the diestrus stage compared to that at the estrus stage in both wild type and TFF1 knock out mice. However, the ER $\alpha$  expression, especially the full-length ER $\alpha$  at 64 kDa, was significantly increased in the hyperplastic uterus from TFF1 knock out mice at estrus and diestrus stages (Fig. 35A), when compared to that of the wild type mice. This indicated that loss of TFF1 led to increased expression of ER $\alpha$ , which might be one of the mechanisms for the hyperplastic uterus.





**FIG. 35. Representative western blot analysis of ER $\alpha$ , and ER $\beta$  expression in the uterus and vagina from the wild type and TFF1 knock out mice**

Three bands of ER $\alpha$  (64, 56, 42 KDa) and ER- $\beta$  (53 KDa) were detected. The ER $\alpha$  expression was slightly increased at the diestrous stage compared to that at the estrous stage in both wild type and TFF1 knock out mice. However, the ER $\alpha$  expression, especially the full-length ER $\alpha$  at 64 kDa, was increased in the hyperplastic uterus from TFF1 knock out mice at the estrous and diestrous stages when compared to that of the wild type mice (A). ER $\beta$  expression was decreased in the uterus of TFF1 knock out mice at the diestrous stage when compared to the wild type mice (B). At the diestrous stage, the ER $\alpha$  expression was significantly increased in the vagina from TFF1 knock out mice when compared to the wild type mice (C). Protein loading was normalized to  $\beta$ -actin using a monoclonal primary antibody at a 1:10,000 dilution, which was specific for a band at 42 kDa. n=2

ER $\beta$  is expressed in the uterus and along with ER $\alpha$  regulates the development of the uterus as well (Hall and McDonnell, 1999). We further investigated the ER $\beta$  expression in the hyperplastic uterus from TFF1 knock out mice. It was shown that, at the estrous stage, the ER $\beta$  expression in the uterus from TFF1 knock out mice was significantly increased when compared with that of wild type mice. However, at the diestrous stage, the ER $\beta$  expression in the uterus of wild type mice was significantly increased when compared to its expression at estrous stage in wild type mice. No obviously increase of ER $\beta$  expression was observed in the uterus from TFF1 knock out mice at the diestrous stage. Therefore, compared to the wild type mice, ER $\beta$  expression was decreased in the uterus from TFF1 knock out mice at the diestrous stage (Fig. 35B), which was inconsistent with the increased ER $\beta$  expression in the uterus from TFF1 knock out mice at the estrous stage. Since 2-3 mice were used at each estrous stage, large samples are still required to confirm the alteration of the ER expression in the uterus from TFF1 knock out mice.

### **3. 3. 2. 7. Loss of TFF1 might result in increased ER $\alpha$ expression in the vagina from TFF1 knock out mice at diestrous stage.**

We also investigated the ER $\alpha$  expression in the vagina from the wild type and TFF1 knock out mice. It appeared that the three bands of ER $\alpha$  were observed in the vagina. However, ER $\alpha$  expression varied in the vagina from the wild type and TFF1 knock out mice at the estrous stage, therefore, it is difficult to determine the impact of loss of TFF1 on the ER $\alpha$  expression at the estrous stage. However, at the diestrous stage, the ER $\alpha$  expression was significantly increased in the vagina from TFF1 knock out mice, when compared to the wild type mice (Fig. 35C), which was consistent with the increased ER $\alpha$  expression in the uterus from TFF1 knock out mice.



## CHAPTER 4: DISCUSSION

### 4.1. Transcriptional regulation of TFF1 by estradiol *in vitro*

#### 4.1.1. The effect of estradiol, ICI 182 780 and tamoxifen on the TFF1 transcription and translation

Estradiol is reported to regulate the transcription of TFF1 (Jakowlew et al., 1984). To investigate the mechanisms underlying TFF1 transcriptional regulation by estradiol, and to establish the contribution of nuclear ERs to TFF1 gene transcription and expression, ICI 182 780 and tamoxifen, the antagonists of estrogen, are used to "knock out" the ER effects. We have shown that  $10^{-15}$  M and  $10^{-5}$  M estradiol were able to induce the transcription of TFF1 in our experiments (Fig. 8A and B). Furthermore, we also provided evidence that tamoxifen has the ability to induce the transcription of TFF1 (Fig. 8 Aand B), which is consistent with the reports that tamoxifen has both agonistic and antagonistic actions (Wakeling, 1993; Brunner et al., 1993) and that it moderately enhances TFF1 mRNA levels in MCF-7 cells (Weaver et al., 1988; Berry et al., 1989). In contrast, Westley et al. (Westley et al., 1984) have reported that tamoxifen could not induce TFF1 gene expression in MCF-7 cells. In their study, we speculate that the induction of transcriptional regulation of TFF1 by tamoxifen may have been masked by the use of a culture medium containing phenol red, which has an estrogen-like effect on the TFF1 transcription (Berthois et al., 1986). ICI 182 780 is classified as a pure antiestrogen (Wakeling et al., 1991), which can't enhance the TFF1 transcription in MCF-7 cells (Osborne et al., 1995). However, in our experiments, ICI 182 780 is able to enhance the TFF1 mRNA level. This is the first study to report the agonistic activity of ICI 182 780 in TFF1 transcriptional regulation. Our result contrasts with the conventional classification of ICI 182 780 as a "pure estrogen antagonist", to block the effects of estradiol and some partial agonists like tamoxifen. ICI 182 780 exhibits no detected agonistic activities in several *in vivo* and *in vitro* models of estrogen action in different mammalian species (Osborne et al., 2004). However, our observation is consistent with recent *in vitro* studies

that reported agonistic or partial agonistic activities for ICI 182 780 (Wu et al., 2005; Robertson et al., 2001; Jones et al., 1999; Wu et al., 2004; Zhao et al., 2006). In addition, it is also reported that in fish, ICI 182 780 mimicked some estradiol actions in gene expression in sea bream liver up-regulating ER $\alpha$ , vitellogenin II, and choriogenin L (Pinto et al., 2006).

The mechanisms for the agonistic effects of ICI 182 780 on the TFF1 transcriptional regulation remain unknown as yet. Most antiestrogens act through competitive binding to ERs, afterwards they induce an inactive conformation of the ligand-dependent AF-2 function of ERs. Their context-specific agonistic activities have been mainly attributed to activation of the ligand-independent AF-1 function or to the induction of a partially active AF-2 conformation (Nettles and Greene, 2005). Possible explanations for the partial ICI 182 780 agonism are: 1) ER activation via non-classical mechanisms (e.g. non-genomic actions and indirect activation at AP-1 promoters); 2) ICI 182 780 activation of other ER subtypes (nuclear ER $\beta$  or membrane ERs) or ER variant proteins whose relative expression depends on the cell type or species (Wu et al., 2005; Wu et al., 2004; Zhao et al., 2006). This was further confirmed by the report that ICI 182 780 is able to promote human ER $\alpha$  interaction with the CBP/p300 in HeLa cells (Jaber et al., 2006). 3) The agonistic effects of ICI 182 780 appeared to be mediated by interaction with membrane-bound receptors (Loomis and Thomas, 2000; Thomas et al., 2006).

ICI 182 780 and tamoxifen can both antagonize the transcriptional effect of estradiol in our experiments, which further confirms that in mammary cells, the effects of estradiol could be antagonized by ICI 182 780 and tamoxifen. However, in our experiment, we showed that  $10^{-8}$  M estradiol can maximally enhance the TFF1 expression at protein level, whereas high concentration of estradiol ( $10^{-5}$  M) is shown to significantly downregulate the TFF1 expression. Tamoxifen is shown to upregulate the expression of TFF1 to almost the same extent as estradiol. In contrast, ICI 182 780 is able to enhance the TFF1 transcription at mRNA level, but downregulate its expression at protein level in cytosol. This observation might result from the translocation of some TFF1 proteins from

cytoplasm to nucleus or blocking the new synthesized TFF1 in the nucleus. The distinct effects of ICI 182 780 on the TFF1 transcription and translation are quite similar to estrogens, which are known to rapidly enhance the ER $\alpha$  and ER $\beta$  transcription at mRNA level, but to downregulate the ER $\alpha$  and ER $\beta$  expression at protein level in several mammalian cell types (Pinzone et al., 2004). This indicates that ICI 182 780 and estrogen share some characterizations on transcription and translation. Since in our experiment, high estradiol concentration ( $10^{-5}$  M) downregulates the TFF1 expression, whereas in the presence of ICI 182 780 and tamoxifen, the downregulatory effect of estradiol is antagonized, leading to upregulation of TFF1 expression. All these data indicate that ICI 182 780 and tamoxifen might compete ER with estradiol.

#### **4.1.2. The effect of E<sub>2</sub>, ICI 182 780 and tamoxifen on the ER $\alpha$ expression**

E<sub>2</sub> regulates target cell proliferation and gene transcription through a pathway of molecular events initiated by the binding of the ER $\alpha$  hormone complex to its cognate DNA target. ER $\alpha$  belongs to the family of nuclear receptors that are ligand-dependent transcriptional transactivators. The classical model for estradiol action proposes that ligand binding induces receptor structural changes resulting in its binding to DNA, followed by the interaction of the estradiol–ER $\alpha$  complex with transcriptional intermediary factors and formation of stable preinitiation complexes, which lead to the initiation of the transcription of the target gene. Therefore, E<sub>2</sub>, ICI 182 780 and tamoxifen might have different effects on the ER $\alpha$  expression during the TFF1 transcription based on their different structures and our observations from our experiments. We have shown that treatment of the cells with estradiol and ICI 182 780 caused a decrease of ER $\alpha$  in the cytoplasm and nucleus (Fig. 8C; Fig. 9), which we think, results from the relocation of ER $\alpha$  from cytoplasm to nuclei where ER $\alpha$  is degraded in a estradiol- or ICI 182 780-dependent fashion. Our results are also in line with the report that treatment with estradiol and ICI 182 780 greatly reduces the receptor levels (Wijayarathne et al., 1999). The reduced ER might result from a rapid degradation of the ER $\alpha$ , since it is demonstrated that the receptor is rapidly degraded in an estradiol-dependent manner through the ubiquitin–proteasome pathway using cells transiently transfected with an

ER $\alpha$ -expressing vector (Nawaz et al., 1999). Furthermore, it is reported that the pure antiestrogen ICI 182 780 reduces the steady-state levels of the ER $\alpha$  by reducing its half-life and increasing the turn over of the ER $\alpha$  (Dauvois et al., 1992), without affecting the ER $\alpha$  mRNA level (Osborne et al., 2004). Other observations indicate that in the presence of ICI 182 780, ER $\alpha$  mobilizes rapidly to an insoluble nuclear fraction (Giamarchi et al., 2002), leading to a decrease of cytoplasmic ER $\alpha$  and soluble nuclear ER $\alpha$ . In contrast, we found that tamoxifen increased ER $\alpha$  in the nuclei in our experiment, accompanied by a decrease of ER $\alpha$  in the cytosol, indicating that tamoxifen might provoke relocalization of the ER $\alpha$  from cytosol to nucleus, which is different from the ER $\alpha$  degradation triggered by ICI 182 780.

#### **4.1.3. Molecular mechanisms underlying the TFF1 transcriptional regulation by E<sub>2</sub>, ICI 182 780 and tamoxifen**

The molecular mechanisms underlying the distinct effects on transcriptional regulation of TFF1 by estradiol, tamoxifen and ICI 182 780 remain unclear. We have shown that there are no or very few competent ER $\alpha$  in the nucleus of MCF-7 cells treated by ICI 182 780. It is demonstrated that ICI 182 780 appears to act at several levels to completely block ER mediated actions, including the competitive inhibition of agonist binding to ER $\alpha$ , the inhibition of ER dimerization, nuclear translocation and transcription activation through both AF-1 and AF-2, and increased ER protein degradation (Osborne et al., 2004). In contrast, in the presence of tamoxifen, ER $\alpha$  is accumulated in the nuclei, allowing it to bind to its target. This accumulation of ER $\alpha$ -tamoxifen complex in the nuclei would lead to the recruitment of the chromatin remodeling machinery to induce the TFF1 transcriptional regulation. Therefore, we speculated that the agonistic effect of tamoxifen on the TFF1 transcription might be a direct consequence of the nuclear pools of ER $\alpha$  available for binding to its DNA target.

Furthermore, it is demonstrated that tamoxifen and ICI 182 780 could induce different conformational changes of the ER $\alpha$ -LBD. A number of studies have provided evidence that individual ligands may induce specific changes in ER conformation (Fritsch et al.,

1992; Beekman et al., 1993). Recent X-ray crystallographic studies demonstrate that there are dramatic differences in the orientation of helix 12 after the ligand-binding domain is occupied by estradiol or raloxifene (Brzozowski et al., 1997). Such changes in receptor conformation could result in the presentation of different functional ER surfaces to form the basis for the recruitment of specific sets of transcription factors to the promoter. This fact suggests that different ER $\alpha$ -ligand complexes may interact with different coactivator/corepressor proteins within the cell to induce or inhibit the transcriptional regulation of target gene (Wijayarathne et al., 1999), which might explain the different transcriptional regulation of TFF1 by E<sub>2</sub>, tamoxifen and ICI 182 780.

In addition, both ER $\alpha$  and ER $\beta$  can interact with the fos/jun transcription factor complex on AP1 sites to stimulate TFF1 gene expression. However, these have opposite effects in the presence of estradiol (Paech et al., 1997). Therefore, typical agonists such as estrogen, and the antiestrogen tamoxifen, function as agonists in the AP1 pathway through ER $\alpha$ . This might be one of the mechanisms underlying the agonistic activity of tamoxifen to transcriptionally regulate the TFF1 gene expression.

Some findings suggest that the agonist/antagonist activity of a given compound is influenced by the cellular expression level of these receptor-associated proteins. For example, overexpression of SRC-1 converts tamoxifen from a pure antagonist to a partial agonist, whereas the overexpression of SRC-1 has no effect on the pharmacology of ICI 182 780 (Smith et al., 1997), which might explain the distinct effects of tamoxifen and ICI 182 780 on TFF1 transcriptional regulation as well.

Furthermore, tamoxifen and ICI 182 780 have very different effects on the interaction of proteins within the TFF1 5' flanking region. It is reported that few proteins are recruited to the TFF1 5' flanking region after tamoxifen treatment of MCF-7 cells, whereas multiple proteins are recruited after treatment of MCF-7 cells with ICI 182 780. The different patterns of protein-DNA interaction observed after treatment of cells with tamoxifen and ICI 182 780 may underlie the distinct transcriptional mechanisms of TFF1 by tamoxifen and ICI 182 780 (Giamarchi et al., 1999).

Our data support the hypothesis that the nature of the bound ligand-like estradiol, ICI 182 780, and tamoxifen, affects the stability and/or the expression level of ER $\alpha$ , and its conformation. This might lead to different activations with coactivators and distinct chromatin remodeling of TFF1.

#### **4. 1. 4. Mechanisms underlying the chromatin remodeling of TFF1 by estradiol**

It is reported that steroid hormones are able to induce hyperacetylation of histones at the promoters of target genes *in vivo*. Evidence shows that hyperacetylation is mediated by receptors and their associated coactivators (Chen et al., 1999). It is defined chromatin histone acetylation as a critical step in nuclear receptor-mediated hormonal signaling because histone acetylation (and deacetylation) plays a critical role in hormone induction of gene expression. Upon hormone binding, nuclear receptors recruit the p160 coactivators and p300/CBP to the target gene. The acetyltransferase activity of p300/CBP will acetylate histones to remodel chromatin and also begin to modify other target proteins such as the p160 coactivators upon hormone induction (Chen et al., 1999). *In vivo* histone acetylation levels of ER and TFF1 target genes are all significantly increased upon estradiol induction.

After being activated by estradiol, the TFF1 gene promoter is packaged into chromatin where two positioned nucleosomes organize the DNA in the region spanning from the CAP site to approximately -420 (Sewack and Hansen, 1997). And the local changes in chromatin structure can be visualized by the induction of hormone-dependent DNase I hypersensitive site (TFF1-HS1) (Giamarchi et al., 2002), which encompasses the ERE (Giamarchi et al., 1999). Treatment of the cells with estradiol for 20 min is sufficient to trigger chromatin remodeling over that region (Giamarchi et al., 2002). Treatment of cells with ICI 182 780 for 2 h results in a significant decrease in DNase I hypersensitivity when compared to an untreated control (Sun et al., 2005). In contrast, treatment of the cells with tamoxifen provokes an increase in the intensity of the DNase I hypersensitivity (Sun et al., 2005), which indicates that estradiol, tamoxifen and ICI 182 780 are able to promote different chromatin remodelings, and therefore leads to different effects on TFF1

transcription.

## **4. 2. Transcriptional regulation of TFF1 by estradiol *in vivo***

Using transgenic rat models to study the transcriptional regulation of TFF1 by estrogen has not been reported previously. In this study, we have successfully generated the *hTFF1-luc* transgenic rat model in which the expression of luciferase reporter gene is successfully directed by the human TFF1 promoter in a tissue specific fashion. The TFF1 promoter is activated by the serum estradiol during the natural estrous cycle.

### **4. 2. 1. Comparison of the advantages and disadvantages between GFP and luciferase reporter genes in transgenic model**

To establish the hTFF1 transgenic rat model, we were able to choose between different reporter genes, including green fluorescent protein (GFP) and luciferase. Therefore, we compared the advantages and disadvantages between the reporter gene of GFP and luciferase in the establishment of the transgenic rat model.

GFP, a 27-kDa monomeric protein from the jellyfish *Aequorea Victoria*, has become tremendously popular as a reporter in fixed and live tissue since the cloning of its gene. EGFP (Haas et al., 1996), is a variant of GFP, in which certain amino acids in the GFP molecule have been altered to shift the excitation spectrum of GFP to longer wavelengths and increase the brightness. The main advantages of GFP are that it can be easily used as a reporter in both live and fixed tissue, and no exogenous substrate is required to express its fluorescence.

However, GFP also presents some limitations and potential disadvantages. The disadvantages of the GFP system are that GFP might interfere with background or nonspecific fluorescence (autofluorescence), and that GFP-positive cells potentially induce immune responses (Stripecke et al., 1999). In addition, the intensity of fluorescence decreases over time, through cell division and differentiation. Furthermore, it is reported that there is substantial variability in the pattern of GFP expression among

mice generated from the same construct (Swenson et al., 2007). Other reports indicate that accumulation of stable GFP induces cell damage or death (Yang et al., 2005). Furthermore, GFP is not suitable for use in specifically studying signaling *in vivo*, because its emission spectrum is not in the longer wavelength part of the spectrum. Only red shifted proteins permit precise deeper tissue imaging because of their spectral properties (Long et al., 2005). The main disadvantage for the GFP is that it is difficult to quantify the reporter gene expression. However, this is required in our experiments, because we have to compare the reporter gene activity to investigate the transcriptional regulation of hTFF1.

Luciferase is a 62-kDa firefly (*Photinus pyralis*) enzyme that catalyzes the reaction of the compound D-(-)-2-(6'-hydroxy-2'-benzothiazolyl) thiazoline-4-carboxylic acid, also known as luciferin, to oxyluciferin in the presence of ATP, O<sub>2</sub>, and Mg<sup>2+</sup>, and produces yellow (560 nm) light as a result. Firefly luciferase is encoded by the *luc* gene. The main advantage of luciferase is that it can be used to detect very low levels of gene expression, because, unlike the fluorescence in the other reporter, the bioluminescence from the reaction it catalyzes is virtually background-free. Furthermore, luciferase expression can be easily quantified and measured by adding luciferin and ATP to cell lysates and then analyzing the emitted light with a luminometer. This exactly satisfies our experimental requirements for quantification of the reporter gene expression to investigate the transcriptional regulation of hTFF1 in our transgenic rats.

The main disadvantage of the luciferase reporter is that it is difficult to deliver luciferin to living animals to detect the luciferase expression. However, this limitation could be overcome by the invention of *in vivo* bioluminescence imaging. Therefore, we select the luciferase as the reporter gene.

#### **4. 2. 2. Tissue specific expression fashion of luciferase directed by hTFF1 promoter**

The 5' flanking region of hTFF1 contains several control elements, which are included



within 1000 bp. These elements are responsive to estrogens, phorbol esters, c-Ha-ras, c-jun, and several growth factors in MCF-7 cells (Nunez et al., 1989). Therefore, we generated a construct with a 1000 bp human TFF1 promoter linked upstream to the luciferase reporter gene in the pGL-3 vectors to establish the transgenic rat.

We have analyzed the tissue specific expression fashion of the *hTFF1-luc* gene to examine whether the 1000-bp hTFF1 promoter is able to direct the expression of luciferase among the organs. We have demonstrated the luciferase tissue specific expression by *in vivo* bioluminescence imaging, which reveals that the luciferase transgene is highly expressed in vagina, followed by heart, brain, uterus and stomach. The method of *in vivo* bioluminescence imaging provides some advantages such as low immunogenicity, negligible toxicity, and the ability to detect the luciferase activity in a living rat. It also makes it possible to compare the luciferase activity among different organs in the same living rat. Therefore, this method is employed in our study to screen the luciferase positive transgenic rats and to detect the tissue expression fashion of the *hTFF1-luc* transgene in the female transgenic rats. The *in vivo* bioluminescence imaging successfully detected the luciferase positive transgenic rats and the luciferase expressing organs in the transgenic rat.

However, the *in vivo* bioluminescence imaging has some limitations. First, light transmission is attenuated approximately 10-fold for every centimeter of tissue through which it passes (Contag and Bachmann, 2002). This fact might explain why the highest luciferase activity tissue in the transgenic rat revealed by *in vivo* bioluminescence imaging is the vagina, not the brain. Because the light emission is attenuated when it passes the head bone during the *in vivo* bioluminescence imaging. Actually, the luciferase activity in brain tissue is the highest, which was further confirmed by *in vitro* luciferase activity assay, RT-PCR and westernblot. For the same reason, photon flux from a superficial organ is greater than that for the same photon flux amount from an internal organ (Contag and Bachmann, 2002), which might explain why the luciferase activity in the vagina from our transgenic rat, revealed by *in vivo* bioluminescence imaging, is much higher than that in

brain and other organs. Secondly, luciferase requires ATP to produce bioluminescence from luciferin (Wilson and Hastings, 1998), so the luciferin in the vessel, vein, heart and extracellular luciferin can't be detected. Finally, the light produced by *in vivo* imaging in organs between adjacent anatomical sites could not be properly distinguished.

However, these limitations can be overcome by using RT-PCR and *in vitro* luciferase activity assay. Thus, combinational use of these three techniques could greatly facilitate the accurate tissue specific expression fashion of the *hTFF1-luc* transgene in the *hTFF1-luc* transgenic rats. RT-PCR revealed that the *hTFF1-luc* gene appeared to be expressed only in the brain, stomach, colon, uterus and vagina, with the highest expression being in the brain. This was confirmed by *in vitro* luciferase activity assay in the female transgenic rats, suggesting that the 1000 bp hTFF1 promoter is able to direct luciferase expression to specific organs. It also indicates that the necessary elements in the hTFF1 promoter to target the luciferase transgene in a tissue-specific expression fashion are located within 1000 bp of 5' flanking sequence in the TFF1 promoter.

#### **4. 2. 3. Possible mechanisms underlying the luciferase tissue specific expression directed by hTFF1 promoter**

The tissue expression fashion of *hTFF1-luc* transgene in the brain, stomach, heart, colon, uterus and vagina seems to be quite similar to the rodent endogenous TFF1 expression fashion, because the rodent endogenous TFF1 is reported to be expressed in the brain, stomach, heart, uterus and vagina as well (Hirota et al., 1994; Wiede et al., 2001; Masui et al., 2006). The observation suggests that hTFF1 promoter is regulated in the same way as the rodent endogenous TFF1 gene. However, the *hTFF1-luc* transgene expression is different in the highest endogenous TFF1 expressing tissues in mouse and human. Because, under physiological conditions, the endogenous TFF1 in mouse and human is highly expressed in the stomach, not in the brain, suggests that the TFF1 promoter is highly activated in the stomach. However, in our *hTFF1-luc* transgenic rat, the *hTFF1-luc* transgene is not highly expressed in the stomach. This might be explained by the fact that the transcriptional factors and regulatory proteins in the rodent stomach are unable to

recognize and bind to the 1000 bp hTFF1 promoter to highly activate the luciferase expression due to the differences between the human and rodent TFF1 promoter. Alternatively, the 1000 bp hTFF1 promoter lacks other important DNA-binding and regulatory elements, which are located up-stream in the promoter, and contribute to highly positive regulation of the *luc* transgene in the stomach tissue *in vivo*.

Also, under physiological conditions, the rodent endogenous TFF1 is synthesized in the central nervous system as well, but to a much lesser extent than in the mucous epithelia in the stomach (Hoffmann et al., 2001), suggesting that the rodent TFF1 promoter is not highly activated by the transcriptional factors in the brain. However, in our female *hTFF1-luc* transgenic rats, we demonstrated that the 1000 bp hTFF1 promoter is highly activated in the brain and cerebellum, suggesting the transcriptional factors in the brain and cerebellum are capable of highly activating the 1000 bp hTFF1 promoter due to the differences between the human and rodent TFF1 promoter. Comparison of the human and mouse TFF1 gene promoter reveals that the mouse TFF1 promoter lacks Motif I and Motif II, which are presented in 1000 bp hTFF1 promoter and play important roles in human TFF1 regulation (Terada et al., 2001). The 1000 bp hTFF1 promoter might be highly activated by the rodent brain- and cerebellum-specific transcriptional factors due to the presence of Motif I, and Motif II, thus leading to a high luciferase expression in brain and cerebellum of the *hTFF1-luc* transgenic rats.

The mechanisms underlying the tissue-specific activation of *hTFF1-luc* transgene are unclear. Generally, the transgene integration occurs randomly, so both the position and structure of the transgenic locus is variable. Position effects are brought about by the influence of local regulatory elements and chromatin structure. The organization of the chromatin around the *hTFF1-luc* transgenic sequences in the luciferase-negative tissues from *hTFF1-luc* transgenic rat appears not to activate the luciferase transgene, or some chromatin adjacent to the integration site may silence the transgene, a phenomenon termed positive effect variegation. Another possibility may be that the 1000 bp hTFF1 promoter lacks other DNA-binding and regulatory elements further up-stream in the

promoter that contribute to positive regulation of the *luc* transgene in the *luc*-negative tissues *in vivo*. Since the hTFF1 promoter is upstream linked to the luciferase gene, luciferase expression might be mainly regulated by the hTFF1 promoter, indicating that tissue-specific regulatory factors are able to activate the hTFF1 promoter in our *hTFF1-luc* transgenic rats, because transcription factors in different cell types may recognize the *hTFF1-luc* transgene differently. In our experiments, it seems that these regulatory factors are only available in the brain, stomach, heart, uterus and vagina.

Furthermore, it is reported that there are some short sequence motifs, (namely, motif I-IV) in the human TFF gene promoter (Gott et al., 1996). The mutagenesis study demonstrates that the motifs III and IV contribute positively to the transcriptional regulation of human TFF1 gene (Beck et al., 1998). In addition, the presence of motif IV in human TFF1 promoter shows striking similarity to the classical binding site of the hepatocyte nuclear factor 3/forkhead (HNF3 /FKH) proteins (Beck et al., 1999), which are transcriptional factors for regulation of tissue-specific gene expression (Lehmann and Korge, 1996). Thus, it is speculated to demonstrate that the HNF-3/FKH factors can recognize human motif IV in TFF1 promoter, trigger the transcription of human TFF1 gene, and contribute to tissue-specific regulation of TFF1 gene (Beck et al., 1999). This might be another mechanism underlying the tissue-specific expression fashion directed by hTFF1 promoter.

However, DNA methylation of the TFF1 promoter is reported to be another mechanism underlying the tissue-specific TFF1 expression (Fujimoto et al., 2000). For example, in the vagina, methylated PCR products are obtained from the HapII-resistant methylated DNA from the vaginal epithelium, suggesting that a methylation of the TFF1 promoter occurred in the vaginal tissue. The amounts, however, are smaller than those in the gastric tissue (Masui et al., 2006). Gastric tissue is reported to be the least methylated tissue with a high level of TFF1 expression, when compared to the highly methylated tissues such as the spleen.

#### **4. 2. 4. The activation of the hTFF1 promoter in brain and the role of TFF1 in the vessel**

Because the brain exhibits the highest luciferase expression in our *hTFF1-luc* transgenic rat model, the brain is used to investigate the location of luciferase transgene in the brain. Immunofluorescence analysis revealed that the luciferase is expressed in the brain vessel from *hTFF1-luc*<sup>+/-</sup> rats at the estrous stage, suggesting that the hTFF1 promoter is activated in the vessel wall of the brain. However, the rodent endogenous TFF1 is reported to be widely expressed throughout the adult brain as well, with high levels in the hippocampus, frontal cortex, and cerebellum (Hirota et al., 1995). In rat brain, TFF1 is detected in astrocytes (Hirota et al., 1994). It is not surprising to find the luciferase expression (hTFF1 promoter is activated) in the rat brain. But it is surprising that the luciferase is not found in the astrocytes, although it is found in the vessel of brain, which suggests that in rat brain, the hTFF1 promoter is not activated in the astrocytes, but in the vessel. This observation might result from the genetic differences between the human TFF1 promoter and mouse TFF1 promoter.

Although we did not identify the specific cell types in which the luciferase is expressed within the vessel wall, we have found that TFF1 is expressed in human microvascular endothelial cells (data not shown). Consistent with our observation, it is reported that TFF2 and TFF3 could be expressed in human vascular endothelial cells as well, especially in vascularized tumor areas and blood vessels adjacent to the primary tumor specimen (Dhar et al., 2005). There, they are likely directly involved in the formation of new blood vessels with implication of cyclooxygenase-2 and EGF receptor signaling during normal and pathophysiological processes linked to wound healing, inflammation, and cancer progression (Rodrigues et al., 2003). This suggests that TFFs are able to function as proangiogenic factors. In addition, it is shown that TFF1 is regulated by estrogen and exerts an anti-apoptotic effect (Emami et al., 2004). Furthermore, estradiol treatment results in a dose-dependent, ER-mediated inhibition of TNF $\alpha$ -induced human endothelial cell apoptosis (Spyridopoulos et al., 1997). Therefore, we speculate that TFF1

might exert an anti-apoptotic effect in the vessel through ER.

#### **4. 2. 5. The activation of the hTFF1 promoter by physiological estrogen during the course of the estrous cycle**

In *in vitro* experiments, we have shown that estradiol is able to regulate the transcription of TFF1 in MCF-7 cells. To further confirm the transcriptional regulation of TFF1 by estradiol *in vivo*, we measured the luciferase level from the *hTFF1-luc* transgenic rats at different estrous stages. The luciferase expression demonstrated that luciferase levels in the brain and cerebellum fluctuate in accordance with physiological estrogen changes during the course of estrous cycle, with a significantly highest luciferase activity in the brain and cerebellum at the proestrous stage and the lowest luciferase activity at the diestrous stage. This might be explained by the fact that the hTFF1 promoter is regulated by estrogen (Emami et al., 2004). At the proestrous stage, we found that the circulating estrogen level in female rats is highest with a concentration of approximately  $88.7 \pm 7.1$  pg/ml and decreased significantly to the lowest level at diestrous with a concentration of  $34.8 \pm 3.2$  pg/ml, almost 3-fold lower than that at the proestrous stage. The fluctuating estrogen level results in a maximal activation of hTFF1 promoter at proestrous and minimal activation of hTFF1 promoter at diestrous. However, no significant elevation of luciferase level is observed at the estrous stage when compared to the diestrous stage. This might be due to the fact that there is no significant difference in the estrogen level at the estrous and diestrous stages, because we find that the estrogen level is  $34.9 \pm 2.6$  pg/ml at the estrous stage, almost at the same level as  $34.8 \pm 3.2$  pg/ml at the diestrous stage.

To further confirm that the increased luciferase in the brain and cerebellum resulted from the activated hTFF1 promoter by the fluctuating serum estrogen, we established a correlation between luciferase activity in the brain and the serum estrogen level at the proestrous and diestrous stages. Indeed, we are able to demonstrate that the luciferase activity is highly correlated with serum estrogen with a correlation coefficient of 0.980, which also further confirmed that the *hTFF1-luc* gene is integral and can be activated by

the serum estrogen.

However, no increased luciferase protein is detected at the proestrous stage in the uterus, vagina, heart, and stomach, when compared to that at the diestrous stage. This might result from the fact that the basal hTFF1 promoter activity is low among these tissues according to the results obtained by RT-PCR and luciferase enzyme activity assay. Thus, the ability of the hTFF1 promoter to respond to estrogen is relatively low and limited. Therefore, it might take longer for the *luc* mRNA to be translated into protein. That might explain why no upregulated luciferase protein is observed in the uterus, vagina, heart, and stomach at the proestrous stage.

We find that the hTFF1 promoter is activated by estrogen in the vagina at the transcriptional level, which is consistent with Masui et al. report that TFF1 promoter is activated in the vagina by estrogen (Masui et al., 2006). They show that the TFF1 mRNA in the vagina is remarkably increased by estrogen in neonatal mice, and pronouncedly intensified expression of the TFF1 gene is observed in the vagina of neonatally estrogenized mice even at adulthood, suggesting that TFF1 might be regulated by estrogen in the vagina. Furthermore, *in vitro* culture of the recombinant epithelium and stroma of neonatally estrogen- and oil-treated mouse vagina shows vaginal hyperplasia regardless of the history of the stroma, concomitant with an increased TFF1 expression, suggesting that estrogen acts directly on the developing vagina with the permanent induction of TFF1 gene expression. It is shown that gene induction does not appear to be related to hypermethylation of the cis-promoter of the TFF1 gene (Masui et al., 2006).

#### **4. 2. 6. The mechanisms underlying the transcriptional regulation of TFF1 by estrogen**

The mechanisms underlying the transcriptional regulation of TFF1 by estrogen are not completely characterized. We have already shown that *in vitro*, the amount of ER $\alpha$  presented in the nuclei is important for the transcription of TFF1 on the basis of its ability to interact with ERE in the TFF1 promoter, suggesting that regulating the amount of ER $\alpha$

in the nucleus is a major action of estrogen with respect to chromatin remodeling and transcriptional control. Upon estrogen induction, it is shown that the ERE in the TFF1 promoter and regions flanking this sequence is extensively protected, which is revealed by DNase I and dimethyl sulfate (DMS) *in vivo* footprinting analysis (Kim et al., 2000). DNase I footprinting further reveals that the consensus ERE half site is protected in the absence of estradiol and that both ERE half sites and adjacent flanking sequences are protected after estrogen treatment. This is consistent with the fact that unoccupied ER is bound to the consensus ERE half site in the absence of estradiol and that an estradiol-occupied ER is bound to both ERE half sites in the presence of estrogen. Since protection of nucleotide sequence adjacent to the consensus ERE half site in the absence of hormone is observed, it is possible that ER binding to the consensus ERE half site could be stabilized by interaction with a protein bound to adjacent nucleotide sequence. The ability of the receptor to bind to the consensus ERE half site in the absence of estrogen could also be fostered by a rather loose association of the ERE-containing DNA with histones (Sewack and Hansen, 1997).

Furthermore, it is reported that a Sp1 binding site positioned next to the ERE in hTFF1 promoter is also important for its transcriptional regulation by estrogen. This is because it is suggested that Sp1/Sp3 contribute to the estrogen-regulated response of the TFF1 gene. In the absence of estradiol, Sp1, Sp3, histone deacetylase 1 (HDAC), and HDAC2, low levels of acetylated H3 and H4 are associated with the native promoter, and the histones are engaged in dynamic reversible acetylation. Following estradiol addition, levels of ER and the steady state levels of acetylated H3 and H4 histones bound to the native hTFF1 promoter increase (Sun et al., 2005). All these observations suggested that differential occupation of the ERE by ER may be involved in silencing, activation, and maintenance of the TFF1 gene expression (Kim et al., 2000). Therefore, the differences in chromatin remodeling induced by estradiol and its antagonist could result from the differences of active ER $\alpha$  in the nuclear pool.

However, the loading of ER onto estrogen-responsive promoters may be direct or indirect.



It is demonstrated that the basal expression of TFF1 mRNA in the M-ERd3/g8 cells, a variant of MCF-7 cell line with a mutant ER lacking the second zinc finger of ER, which is required for direct DNA interaction, falls 50-fold compared with that in MCF-7 cells (Pentecost et al., 2005). This suggests that ER is likely to directly bind to ERE to activate the TFF1 promoter.

Furthermore, it is reported that estrogen is able to indirectly regulate the TFF1 transcription, since estrogen-induced secretion of growth factors may play an important role in the mouse TFF1 transcriptional regulation by estrogen. It is reported that the transcriptional regulation of TFF1 by estrogen may involve an estrogen-induced EGF secretion (Masui et al., 2006). EGF is one possible mediator of estrogen-induced TFF1 expression in the proliferative epithelium. EGF regulates the transcription of TFF1 because an EGF-responsive enhancer region is demonstrated to be contained in the 5'-flanking region of TFF1 promoter (Terada et al., 2001).

In addition, estrogens may also induce the expression of the c-fos proto-oncogene, which is known to cooperate with the AP1/cjun protein in enhancing transcription of TFF1 (Sassone-Corsi et al., 1988). Thus, in addition to its primary response to estrogens, the response of TFF1 enhancer region to several protein factors, which are known to mediate the mitogenic activity of estrogens (Nunez et al., 1989), might also play an important role in the mechanisms underlying the transcriptional regulation of mouse TFF1. It is necessary to have transgenic rats ovariectomized to get rid of the effect of endogenous estrogen, and administration of estradiol to investigate the different mechanisms underlying the transcriptional regulation of hTFF1 and endogenous TFF1 by estrogen *in vivo*.

Taken together, our data indicate that the *hTFF1-luc* transgenic rat model is successfully established and the physiological changes in circulating gonadal steroids such as estrogen can significantly regulate hTFF1 promoter, and thus can affect the relative level of luciferase. The *hTFF1-luc* rat model with the tissue-specific expression of the *hTFF1-luc* transgene, offers a great opportunity to investigate the hTFF1 transcriptional

regulation and its possible mechanisms by estrogen and other regulatory factors *in vivo*. Since TFF1 is an estrogen-responsive gene, this transgenic rat model is useful in toxicology to efficiently and accurately screen some xenobiotic substrates or some metabolites of xenobiotic substrates in an *in vivo* context, which are suspected of exerting an estrogen-like effect on transcriptional regulation of TFF1 gene *in vitro*.

#### **4. 3. Biological function of TFF1 in the female reproductive organs**

Most of the studies on biological functions of TFF1 are investigated mainly on the gastrointestinal tract. Its functions in the female reproductive organs are poorly understood. In this study, we have first shown that loss of TFF1 leads to hyperplastic uterus and increased total epithelial thickness in uterus and vagina in mice by downregulation of E-cadherin expression. This is the first direct evidence to unravel the biological function of TFF1 in uterus and vagina.

##### **4. 3. 1. The biological functions of TFF1 in the uterus and vagina**

We have shown that morphological alterations in uterus and vagina are observed in 12-month-old TFF1 knock out mice. The uterus from TFF1 knock out mice is much bigger than that of wild type mice (Fig. 22), with a two-fold increased ratio of relative uterine weight (uterus to body weight) and an increased uterine diameter. However this alteration in the uterus is not observed in 5-month-old TFF1 knock out mice (Lefebvre et al., 1996), which might suggest that TFF1 is not required for the uterine development and morphogenesis, but might be required for maintaining normal uterine morphology later in life. The hyperplastic uterus from the TFF1 knock out mice stimulated us to further examine the uterine and vaginal histology. We have provided the evidence that loss of TFF1 resulted in a significantly increased epithelial height and the increased thickness of total uterine and vaginal epithelium at estrous and diestrous, which is in line with the report that hyperplastic alteration is observed in stomach from the TFF1 knock out mice, because the antral and pyloric mucosa in the stomach of TFF1 knock out mice is thicker. The mucosa of a 3 week-old pup is already twice the normal thickness and the antral and

pyloric mucosa shows severe hyperplasia. The epithelial cells lining the surface and the elongated pits show high-grade dysplasia in TFF1 knock out mice. In addition, the antral and pyloric epithelial cells are improperly differentiated and dysfunctional (Lefebvre et al., 1996). Our observations suggest that TFF1 plays an important role in the control of epithelial cell proliferation and differentiation in the uterus and vagina.

Furthermore, in human endometrium, it is reported that TFF1 is intensively expressed in the human glandular epithelium of the endometrium. The high level of TFF1 expression is observed throughout the menstrual cycle in 90 percent of the cases studied (Rye et al., 1993). In contrast, no expression or very weak expression of TFF1 is detected in endometrial adenocarcinomas (Henry et al., 1991; Wysocki et al., 1990), which further supports the possible function of TFF1 as a regulator of cell proliferation in uterus. This function of TFF1 is supported by its structural similarity to several peptide growth factors such as insulin-like growth factor-1 and porcine spasmolytic polypeptide (PSP, TFF2) (Baker, 1988; Mori et al., 1988). To further confirm the biological function of TFF1 as a regulator of cell proliferation, we examined the uterine and vaginal epithelial cells in the TFF1 knock out mice by electron microscopy analysis. And loss of cell-cell contact and cell-cell adhesion between the uterine and vaginal epithelial cells are observed, which further confirmed the biological function of TFF1 as a regulator of cell proliferation.

Another proposed biological function of trefoil factors is the regulation of the regeneration of the human endometrium following menstruation (Rye et al., 1993). In human, proliferative endometrium needs a number of growth and remodeling factors for successful regeneration of the denuded endometrium. Because the wound-healing process after menstruation begins with the re-epithelialization of the uterine surface, indicating that some factors might be involved in epithelial repair. The trefoil family of peptides (TFF) might be one of the candidate factors, since TFFs are reported to be expressed in the human uterus (Wiede et al., 2001). Specifically, TFF3 mRNA is in the surface epithelium of the endocervix. The increased level of TFF3 mRNA during menstrual repair and epithelial proliferation suggests that this factor may play a role,

because considerable epithelial cell migration is required at this time. TFF1 and TFF2 mRNA are detected occasionally in the endocervix and rarely in the endometrium. TFF1 mRNA expression is presented in trace amounts in the human endocervix which might be due to a low epithelium/stromal cell ratio in the tissues analyzed (Rye et al., 1993). Thus, TFF1 might promote repair of the endometrium by action of its mitogenic effect.

The other proposed biological function of TFF1 in the human uterus is to influence the rheological properties of mucous gels together with mucin (Wiede et al., 2001). Synthesis of secretory mucins is typically accompanied by co-secretion of TFF-peptides (Wright et al., 1997). Three human TFF-peptides have been characterized as constituents of mucous gels (Hoffmann et al., 2001; Hoffmann and Hauser, 1993). The mucus lining of the female reproductive tract is important for its defense and physiological function. It inhibits invasion by microorganisms. The rheological properties of the cervical mucus are primarily influenced by estrous cycle and controls sperm entry into the uterine cavity (Wolf and Litt., 1986). Sperm survival and penetrability are maximal when the viscoelastic properties reach a minimum during the ovulatory phase. TFF concentrations play a major role influencing the rheological properties of the endocervical mucus, which is further confirmed by the preliminary studies by the ability of TFFs to increase the viscosity of mucin preparations (Dignass et al., 1994). Therefore, TFFs might influence the female reproductive capacity by influencing the rheological properties of the endocervical mucus. In our experiments, we tried to breed 6 pairs of 12-month-old male and female TFF1 knock out mice, only 1 female was able to get pregnant and had offspring with only 1-2 pups per litter. The other 5 female mice were unable to get pregnant, which supports the proposed function of TFF1 in the uterus, which is to influence the rheological properties of the endocervical mucus, and thus influence the female reproductive capacity.

The biological functions of TFF1 in the vagina are rarely investigated. We first provided the evidences to show that loss of TFF1 leads to the hyperplasia of vaginal epithelial cells, which further confirmed the physiological role of TFF1 in control of the epithelial cell proliferation. TFF1 is proposed to be a useful marker for developmental estrogenization

syndrome of the mouse vagina (Masui et al., 2006).

#### **4. 3. 2. The impact of loss of TFF1 on the E-cadherin expression**

We have shown by electron microscopy analysis that the uterine and vaginal epithelial cells from the TFF1 knock out mice lost the cell-cell contact between the neighboring cells, and uterine and vaginal epithelial cells appeared to lose the cell adhesive junction with the neighboring epithelial cells (Fig.29). As in epithelial cells, cell-cell adhesion is mediated primarily by E-cadherin, a 120-KDa transmembrane glycoprotein localized at the adherens junctions (Takeichi, 1991). In the presence of  $Ca^{2+}$ , E-cadherin's extracellular domain interacts homotypically with the E-cadherin's molecules of neighboring cells to maintain intercellular adhesion, and its cytoplasmic carboxy tail associates with a group of closely related, but distinct membrane undercoat proteins, termed the catenins ( $\alpha$ ,  $\beta$ , and  $\gamma$ )(Gumbiner and McCrea, 1993). Both  $\beta$ -catenin, and  $\gamma$ -catenin bind directly to the cytoplasmic domain of E-cadherin, and  $\alpha$ -catenin links the  $\beta$ -, and  $\gamma$ -catenin to the actin microfilament network of the cellular cytoskeleton (Nieset et al., 1997). Such binding is essential for the adhesive function of E-cadherin and for the establishment of tight physical cell-cell adhesion (Shimoyama et al., 1992).

In addition, it is demonstrated that perturbation of E-cadherin-catenin-mediated cell adhesion is shown to be associated with cell migration and epithelial restitution in an *in vitro* model of epithelial injury (Hanby et al., 1996). *In vivo*, the regenerating epithelium over ulcerated mucosa shows loss of membranous localization and decreased levels of E-cadherin and  $\alpha$ -catenin. Therefore, based on the observation of loss of cell-cell contact and cell-cell adhesion in the uterine and vaginal epithelial cells, and the biological function of E-cadherin as primary mediator of the cell-cell adhesive, we speculate that TFF1 interacts with E-cadherin, which stimulated us to perform *in vitro* transient transfection assay to investigate the effect of TFF1 on the E-cadherin regulation.

We have shown that TFF1 alone is unable to activate the E-cadherin promoter, but in the presence of ER  $\alpha$ , TFF1 significantly activates the E-cadherin promoter (Fig. 10). To

further confirm the upregulatory effect of TFF1 on E-cadherin, we examined the expression of E-cadherin in the uterus and vagina from the TFF1 knock out mice, which are the female reproductive organs with high ER  $\alpha$  expression. We found that loss of the TFF1 leads to downregulation of E-cadherin in the uterus and vagina by western blot, immunofluorescence and real-time PCR, when compared to wild type mice. These observations further reinforced our notion that loss of TFF1 results in a downregulation of E-cadherin, which in turn leads to the loss of cell-cell contact or cell-cell adhesion in the uterine and vaginal epithelial cells in the TFF1 knock out mice. However in our study, we did not find the downregulated expression of  $\beta$ -catenin in the uterus and vagina from the TFF1 knock out mice.

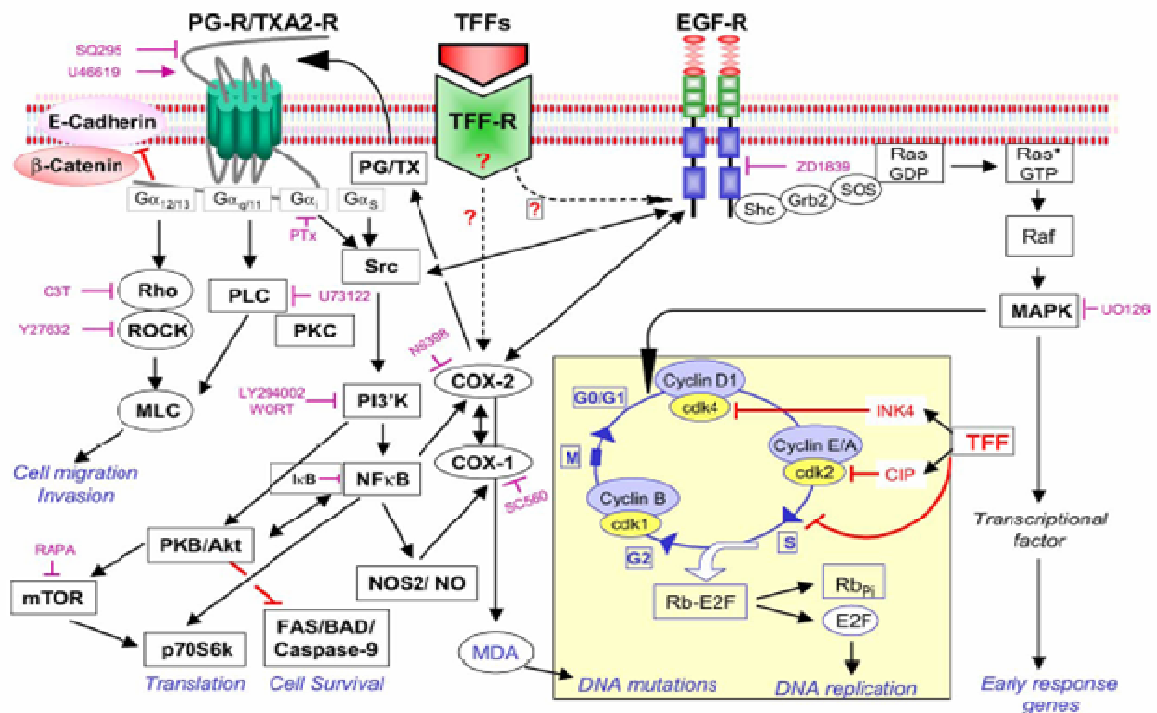
The effect of TFFs on the E-cadherin expression was previously reported. However, our results are not in agreement with previous reports that TFF3 treatment leads to reduced E-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin in colorectal carcinoma cells *in vitro* (Efstathiou et al., 1998). In TFF3-transfected cells, the amount of E-cadherin is demonstrated to be reduced, accompanied by the reduction of  $\alpha$ - and  $\beta$ -catenin levels *in vitro* (Meyer zum et al., 2004), which is in contrast to our observations that loss of TFF1 results in reduced expression of E-cadherin not accompanied by alteration of  $\beta$ -catenin. This might result from the differences between physiological and pathological conditions. In our experiments, we used the physiological mice, instead of the carcinoma cells in their experiments, because it is demonstrated that TFF1 exerts distinct biological functions under different conditions. TFF1 is described to be a stomach-specific tumor suppressor gene under the physiological conditions (Lefebvre et al., 1996), whereas TFF1 functions as tumor progression factors, proangiogenic factors, and promotes tumor cell invasion under the pathological conditions (Prest et al., 2002; Rodrigues et al., 2001). In addition, the differences between *in vivo* and *in vitro* environment might result in different alteration of E-cadherin and  $\beta$ -catenin. Different cellular environments and single and multi-cell types could influence the expression of E-cadherin and  $\beta$ -catenin. However, our results are in line with the report that overexpression of TFF3 in human colon carcinoma cells reduced cellular growth *in vitro* and *in vivo*, and modestly reduced EGF-induced

phosphorylation of MAPKs in the TFF3-expressing clones, suggesting that TFF3 may function as an inhibitory factor for the growth of colonic neoplasm (Uchino et al., 2000).

#### **4. 3. 3. Mechanisms underlying the interaction between TFF1 and E-cadherin**

So far, little is known about the mechanisms underlying loss of TFF1 leading to downregulation of E-cadherin. To better understand the mechanisms, we referred to the signal transductive pathway, which is still poorly understood and very complex (Fig. 31). TFF1 peptide signaling pathways seem to interact with cyclooxygenases (COX-1 and COX-2), and the heterotrimeric G-protein coupled receptors (GPCR): prostaglandin receptor (PG-R) and thromboxane A2 receptor (TXA2-R). COXs produce prostaglandins (PG) and TXA-2 (TX) acting through their corresponding GPCR. The TXA-2-R activation then transmits signals to the heterotrimeric G proteins subunits:  $G\alpha_{12/13}$  that activates the Rho GTPase and E-cadherin signaling pathway, and subsequently cytoskeletal dynamics (Emami et al., 2004). Therefore, loss of TFF1 might modulate the expression of E-cadherin through these signaling transduction pathways. In addition, it is shown that the TFF1 has the potential to transactivate the EGF receptor. And it is demonstrated that rapid tyrosine phosphorylation of  $\beta$ -catenin and enhanced association with the EGF receptor results in loosening of intercellular adhesion similar to EGF-induced tyrosine phosphorylation (Hoschuetzky et al., 1994). The induction of tyrosine phosphorylation of  $\beta$ -catenin is associated with cellular redistribution or loss of E-cadherin (Liu et al., 1997). However, transcriptional repressor proteins of E-cadherin such as Snail, Slug, SIP1 and E12/E47 attract much attention in the regulation of the expression of E-cadherin. Therefore, loss of TFF1 might transcriptionally upregulate such repressors. Furthermore, it is demonstrated that in hTFF3 transfected cells, the half-life of E-cadherin is reduced, suggesting that enhanced proteolytic degradation either by increased rates of endocytosis or shedding of the E-cadherin extracellular domain may contribute to the reduction of the amount of E-cadherin at the cell surface. And it was recently shown that tyrosine phosphorylation of E-cadherin may result in increased endocytosis and reduction in E-cadherin half life (Fujita et al., 2002), or other mechanisms leading to transcriptional

repression of E-cadherin may be activated by the loss of TFF1 in the TFF1 knock out mice.



**FIG. 36.** Proposed model for TFF signal transduction pathways in neoplastic progression and tumor cell invasion (Emami et al., 2004).

#### 4. 3. 4. Mechanisms underlying loss of TFF1 leading to uterine and vaginal hyperplasia

The mechanisms underlying loss of TFF1 leading to hyperplasia in the uterus and vagina are not clear. First, we speculate that the hyperplasia might be due to the loss of TFF1, as loss of TFF1 is reported to result in intense proliferation and defective differentiation of the gastric epithelial cells, showing that TFF1 may be involved in cell growth arrest and/or early differentiation processes occurring during the homeostasis of tissues (Lefebvre et al., 1996). This suggests that TFF1 might contribute to the control of the cell proliferation. Furthermore, the loss of TFF1 leads to the development of the gastric carcinomas (Lefebvre et al., 1996) and the lack of TFF1 expression in some human gastric carcinomas (Machado et al., 1996; Henry et al., 1991) is in favor of a gastric-specific



tumor suppressor gene function for TFF1. However, in our experiments, no carcinogenesis was observed in the uterus or vagina from TFF1 knock out mice.

In addition, we speculate that the downregulation of E-cadherin is involved in the uterine and vaginal hyperplasia process. E-cadherin not only plays an important role in the cell adhesion system, but also acts as a tumor and invasion suppressor and regulates cell proliferation, as E-cadherin has been reported to inhibit cell proliferation by a mechanism which includes upregulation of the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup> (St et al., 1998). Furthermore, it is demonstrated that the growth-suppressive function of E-cadherin is impaired by tumor-associated E-cadherin mutations (Fricke et al., 2004). All the observations indicate that the reduced expression of E-cadherin might result in the hyperplasm in the tissues.

It has been shown that E-cadherin regulates cell growth by modulating the  $\beta$ -catenin transcriptional activity (Stockinger et al., 2001), as E-cadherin is able to form the complex with  $\beta$ -catenin, which has been shown to serve as a key component in signaling processes during embryonic development and adult tissue homeostasis through a Wnt signal transductional pathway. In the absence of Wnt ligand, cytoplasmic  $\beta$ -catenin is NH2-terminally phosphorylated by glycogen synthase kinase 3 $\beta$  (Ikeda et al., 1998), which targets the protein for proteosomal degradation (Orford et al., 1997). The binding of Wnt to its cognate receptor triggers a cascade of signaling events that leads to the accumulation of hypophosphorylated  $\beta$ -catenin in the cytoplasm and the nucleus, and interacts with high mobility group box containing transcription factors of the lymphoid enhancer binding factor (LEF)-1/T cell factor (TCF) family (Huber et al., 1996), which can transactivate transcription by binding to coactivators or can repress transcription by interaction with repressors. Therefore, the  $\beta$ -catenin-LEF/TCF complex is highly regulated and controls the expression of a diverse set of target genes during development and tissue formation (Roose and Clevers, 1999). Thus, inappropriate activation of  $\beta$ -catenin-LEF/TCF complex is shown to drive cell proliferation and tumor formation (Polakis, 2000), which is thought to turn on the expression of c-myc, and the

cell cycle regulator cyclin D1 (He et al., 1998).

However, in our experiments, no altered expression of  $\beta$ -catenin is observed in the uterus and vagina from TFF1 knock out mice. The immunofluorescence analysis revealed no translocation of E-cadherin or  $\beta$ -catenin to the cytoplasm and nucleus, except for the reduced expression of E-cadherin that was detected in uterus and vagina from the TFF1 knock out mice. All the observations appear not to be related with the  $\beta$ -catenin-LEF/TCF complex activity. However, it is demonstrated that E-cadherin is able to decrease  $\beta$ -catenin-LEF/TCF activity and negatively to affect cell proliferation in a cell adhesion-independent manner (Stockinger et al., 2001). Therefore, the activity of  $\beta$ -catenin-LEF/TCF seems to be controlled by E-cadherin in a cell adhesion-independent manner. Loss or downregulation of E-cadherin expression is able to induce cell proliferation by enhancing  $\beta$ -catenin/LEF activity. In our study, we found that the E-cadherin expression was reduced in the uterus and vagina from TFF1 knock out mice. Therefore, activity of  $\beta$ -catenin-LEF/TCF might increase, which in turn results in uterine and vaginal epithelial cell proliferation. This might be one of the main mechanisms underlying the hyperplastic uterine and vaginal epithelial cells, although the expression of  $\beta$ -catenin is not decreased.

In addition, it is shown that the TFF1 has the potential to transactivate the EGF receptor, which in turn activates several downstream effectors, such as PI3'-kinase/PKB/Akt and the Grb2/SOS/Ras/Raf/MAPK cascade. These effectors influence cell survival and cell cycle progression (Emami et al., 2004). Therefore, through this pathway, loss of TFF1 might result in the loss of control of the cell cycle and lead to the uterine and vaginal hyperplasm in the TFF1 knock out mice.

The hyperplastic uterus and the increased epithelial cells in the vagina could possibly be provoked by a dysregulation of ER $\alpha$  and ER $\beta$ . We have also shown that the uterine diameter is significantly increased in the TFF1 knock out mice accompanied by the hyperplastic stroma cells, where no TFF1 but ER $\alpha$  and ER $\beta$  are expressed. And the effects of estrogens on the uterus and vagina are predominantly mediated by ER, which

is fundamental for the development and growth of the uterus and vagina. Therefore, we hypothesized that loss of TFF1 might influence the expression of ER $\alpha$  and ER $\beta$ , which in turn results in the development of hyperplastic uterus. To verify that hypothesis, western blot analysis was performed to quantify the expression of ER $\alpha$  and ER $\beta$  in the hyperplastic uterus and the vagina. We have shown that ER $\alpha$  is slightly increased in the hyperplastic uterus from TFF1 knock out mice at estrous and diestrous stages (Fig. 35 A) when compared to wild type mice. Our observation of the increased expression of ER $\alpha$  in the uterine hyperplasm in TFF1 knock out mice is consistent with previous reports showing that disrupted or mutated ER $\alpha$  resulted in the diminished size of stromal, epithelial, and myometrial tissue compartments in the uterus (Lubahn et al., 1993). This indicates that increased expression of ER $\alpha$  might play an important role in the uterine hyperplasm. As ER $\beta$  is expressed in the uterus and contributes to the regulation of uterine growth as a modulator of the ER $\alpha$ -mediated effects in the uterus (Weihua et al., 2000), we further examined its expression and found that ER $\beta$  expression was increased at estrous, and decreased at the diestrous stage in the uterus from TFF1 knock out mice. This suggests that ER $\beta$  expression varies in the uterus from TFF1 knock out mice. However, compared with ER $\beta$ , ER $\alpha$  is more critical for normal uterine development and growth, because it is reported that in the ER $\alpha$ -disrupting mice, obviously altered reproductive function is observed (Lubahn et al., 1993), while ER $\beta$  deficiency did not perturb uterine growth or function (Krege et al., 1998). Furthermore, in our experiment, ER $\alpha$  expression in the vagina from knock out mice varies at the estrous stage, whereas at the diestrous stage, increased ER $\alpha$  expression is detected. More mouse samples are still required to confirm the role of ER in the hyperplastic uterus and vagina from the TFF1 knock out mice.

The mechanisms through which ER regulates the development and growth of uterus have not been completely characterized. Some authors find that isolated uterine epithelial cells cultured *in vitro* do not proliferate in response to estrogens (Cunha et al., 1983; Cooke et al., 1986; Pierro et al., 2001), thus showing the need for additional trophic factors. This critical observation suggests that proliferation of endometrial epithelial cells

might be stimulated through a paracrine mechanism by growth-promoting substances elaborated in stromal cells in response to estrogen (Cunha et al., 1983; Cooke et al., 1986; Pierro et al., 2001). Similar conclusions are reached using tissue recombination experiments, showing that epithelial mitogenesis in response to estrogen required ER-expressing stromal cells (Cooke et al., 1997). These results indicate that stromal tissue is the source of putative estrogen-dependent growth factors responsible for mediating proliferation of endometrial epithelial cells in response to estrogen treatment, which is in line with the reports that growth factors secreted by stroma in response to estrogen are the mitogens in the epithelium (Cooke et al., 1997). Our results appear to support this observation because hyperplasm of both epithelial cells and stroma cells is observed in the uterus from TFF1 knock out mice. However, further investigations are still required to address the mechanisms underlying loss of TFF1 leads to the altered ER expression.

In the vagina, it is reported that treating the developing vagina with estrogen leads to permanent hyperplasia of the epithelium concomitant with increased expression of TFF1 (Masui et al., 2006), which seems to conflict with our proposed function of TFF1 to control the epithelial cell proliferation in the vagina. This might be explained by the fact that the function of TFF1 to control of epithelial cell proliferation could be antagonized by the ability of estrogen to induce the hyperplasm of vaginal epithelial cells. Treating the developing vagina with estrogen leads to permanent hyperplasia of the epithelium, and it is reported to involve complex epithelium-stroma interaction, which can be divided into three steps. At the first step, exposure of the vagina to estrogen induces persistent changes in the epithelium, which possibly is mediated by keratinocyte growth factor receptor signaling (Hom et al., 1998; Masui et al., 2004). Consequently, at the second step, the abnormally differentiated epithelium in turn induces irreversible changes in stromal development so that, at the third step, the stroma assures permanent proliferation of the epithelium regardless of the presence of estrogen (Masui et al., 2006). In our experiments, the hyperplastic epithelial cells in the vagina resulting from loss of TFF1 might also involve the interaction of stroma cell. This may be true, because studies of the

gastric tissue from TFF1 knock out mice suggest that the presence/absence of epithelial TFF1 influences the physiology and gene expression of the stroma and, possibly, also stromal cell differentiation (Saukkonen et al., 2003). EGF is shown to be one possible mediator of estrogen-induced TFF1 expression in the proliferated vaginal epithelium. The 5'-flanking region of TFF1 contains an EGF-responsive enhancer region that mediates EGF-induced expression of TFF1 in the gastric tissue (Terada et al., 2001a), whereas EGF-EGFR signaling is activated in the transiently or permanently hyperplastic epithelium of the estrogenized vagina (Miyagawa et al., 2004).

#### **4. 3. 5. Proposed mechanisms underlying loss of TFF1 results in the impaired reproductive capacity in the female mice.**

Impaired female reproductive capacity is observed in the TFF1 knock out mice. This is shown through our experiments in which among six pairs of inbreeding mice, 5 pairs of 12-month-old female TFF1 knock out mice could not get pregnant. Furthermore, one pair of breeding mice had a very small size litter with 1-2 pups. The possible mechanisms underlying loss of TFF1 results in the impaired reproductive capacity in the female mice might include: 1) Irregular estrous cycle length, which is observed in the 12-month old female TFF1 knock out mice. Most of the female mice have a prolonged estrous cycle of 6-9 days with 4-6 days at the diestrous stage or 3-6 days at the proestrous stage. Some female mice skip the estrous stage, switching from the proestrous stage directly to the diestrous stage, which suggests that ovulation phase might be influenced. 2) Loss of microvilli and cilia on the uterine surface of TFF1 knock out mice, which is revealed by the electron microscope analysis in our experiment. The uterine microvilli and cilia are important for the fertilized eggs transportation from oviduct to uterus for implantation. 3) The hyperplastic uterine epithelial cells. To keep normal uterine epithelial cells is essential for uterine receptivity for blastocyst attachment, because it was demonstrated that penetration of the uterine epithelial basement membrane is important during blastocyst implantation in the mouse (Blankenship and Given, 1992). 4) The altered or influenced rheological properties of mucous gels in the uterus, because TFFs together with mucin

are reported to influence the rheological properties of mucous gels (Wiede et al., 2001), which control sperm entry into the uterine cavity. Sperm survival and penetrability are maximal when the viscoelastic properties reach a minimum during the ovulatory phase.

## CHAPTER 5: Summary

Although the ability of estradiol to regulate the human TFF1 (estrogen responsive gene) expression is established *in vitro*, the underlying mechanisms remain undefined. In this thesis, we examined and investigated the transcriptional regulation of human TFF1 by E<sub>2</sub> and its antagonists ICI 182 780 and tamoxifen in MCF-7 cells. We presented evidence to show that ICI 182 780 and tamoxifen, like E<sub>2</sub>, were able to activate the human TFF1 promoter and up-regulate its expression. Especially, this was the first time to show that ICI 182 780 had an agonist effect on TFF1 promoter in MCF-7 cells. We observed in our experiments that the transcriptional regulation of TFF1 by E<sub>2</sub>, ICI 182 780 and tamoxifen was through down-regulation of ER $\alpha$  in nucleus, whereas tamoxifen regulated the TFF1 expression by upregulation of ER $\alpha$  in nucleus, indicating different chromatin remodeling and transcriptional mechanisms between ICI 182 780 and tamoxifen.

After examination and investigation of the transcriptional regulation of TFF1 by E<sub>2</sub> and its antagonists ICI 182 780 and tamoxifen *in vitro*, we further attempted to investigate the transcriptional regulation of TFF1 by estrogen *in vivo*. hTFF1 promoter had to be introduced into the animal model. Therefore, we generated a transgenic rat model harboring the human TFF1 promoter linked to a luciferase reporter gene to explore regulation of the TFF1 by estrogen, especially in the female reproductive system. Three lines of *hTFF1-luc* transgenic rat model were successfully established and further characterized. *In vivo* bioluminescent imaging revealed that the transgene of luciferase was mainly detected in the brain, cerebellum, heart, stomach, uterus and vagina. RT-PCR revealed that the unique expression of luciferase was localized in the tissues of the brain, heart, stomach, colon, uterus vagina and ovary, with the highest luciferase mRNA level in the brain. This was later further confirmed by luciferase activity assay *in vitro* which was 25-fold higher in the brain than in stomach in female heterozygous transgenic rats, suggesting that hTFF1 promoter was able to direct the expression of luciferase transgene in a tissue-specific fashion mainly in the brain, and cerebellum. In the brain, luciferase was detected to localize to the vessel by immunofluorescence. Most

important, we further found that the hTFF1 promoter can be activated by the physiological estrogen in female homozygous transgenic rats during the course of estrous cycle. It was shown that at the proestrous stage the luciferase activity in the brain and cerebellum was approximately 3.7-fold and 3-fold higher, respectively, than at the diestrous and estrous stages. Also, the luciferase activities in the heart, stomach, uterus and vagina were, as a whole, increased at proestrous as well, but did not reach a statistical significance, which was further confirmed by westernblot analysis. Real-time PCR revealed that the luciferase mRNA in the brain and cerebellum at the proestrous stage was 2.8-fold and 3.3-fold higher, respectively, than that at the diestrous stage. However, the luciferase mRNA in the heart, stomach, uterus and vagina was significantly increased by 4-, 7-, 3.8- and 11-fold, respectively, when compared to the corresponding tissue from diestrous rats, indicating a lag time to the activation of hTFF1 promoter at protein level in these tissues. All these data demonstrated that the hTFF1 promoter was able to direct the expression of the luciferase reporter gene specifically to the brain, cerebellum, heart, stomach, uterus and vagina in female transgenic rats and that the physiological changes in circulating estrogen can significantly regulate hTFF1 promoter and affect the relative level of luciferase. This *hTFF1-luc* rat model offered an opportunity to investigate the hTFF1 transcriptional regulation and its possible mechanisms *in vivo* by estrogen and other regulatory factors. This rat model is useful in toxicology for efficiently and accurately screening some xenobiotic substrates or some metabolites of xenobiotic substrates in an *in vivo* context, which are suspected of exerting an estrogen-like effect to transcriptionally regulate the hTFF1 promoter *in vitro*.

The biological functions of TFF1 are still not well understood. To date, most research on TFF1 has concentrated on its role in the gastrointestinal wound healing process and the promotion and migration of gastrointestinal carcinomas. Loss of TFF1 expression has been observed in the majority of human gastric cancers and the biological significance in the gastrointestinal tract of this loss has been demonstrated using the TFF1 knock out mouse model. However, the biological significance of TFF1 in the female reproductive system is poorly understood. To understand the biological role of TFF1 in the uterus and



vagina, we investigated the effect of the loss of TFF1 on gross morphology and the expression of E-cadherin (a prime mediator of  $\text{Ca}^{2+}$  dependent cell-cell adhesion) in the uterus and vagina of 12-month-old TFF1 knock out mice. Striking morphological alterations were observed. The uterus from TFF1 knock out mice was much bigger than that of wild type mice with a much higher ratio of relative uterine weight (uterus to body weight). The thickness of the total uterine and vaginal epithelial cells was significantly increased. The stroma cells exhibited much hyperplasia and the uterine diameter was significantly increased when compared to that of wild type mice, suggesting the uterus manifested severe hyperplasia. Electron microscopy analysis revealed that the uterine and vaginal epithelial cells lost the cell-cell contact and cell-cell adhesion with the neighboring epithelial cells. Immunofluorescence, real-time quantitative PCR and western blot analysis revealed that the expression of E-cadherin but not  $\beta$ -catenin, was downregulated in the uterus and vagina of TFF1 knock out mice when compared to the wild type mice. Abnormal expression of  $\text{ER}\alpha$  and  $\text{ER}\beta$  in the uterus and vagina from TFF1 knock out mice might be involved in the alteration process, which needs to be further confirmed. Our data first clearly indicated that the loss of TFF1 in 12 month-old mice induced uterine hyperplasia and increased total thickness of uterine and vaginal epithelial cells by downregulating the expression of E-cadherin, but not  $\beta$ -catenin. This suggests that TFF1 might play an important role in the regulation of cell differentiation and proliferation in the uterus and vagina. Further studies are still required to address the specific molecular mechanisms on E-cadherin and ER regulation by TFF1.

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## 7. Abbreviations

AF- activation function  
AP1- activator protein 1  
bFGF - basic fibroblast growth factor  
CMR-complete mini reagent  
COX-cyclooxygenases  
DBD- DNA binding domain  
DEPC-diethylpyrocarbonate  
DMS - dimethylsulfate  
E2 - estradiol  
EGF - epidermal growth factor  
EGFP-Enhanced green fluorescent protein  
EGFr - epidermal growth factor receptor  
ER - estrogen receptor  
ERE - estrogen-responsive element  
FGF- fibroblast growth factor  
FKH-forkhead  
GFP-green fluorescent protein  
GPCR-G-protein coupled receptors  
HDAC-histone deacetylase  
h-hour  
HNF3-hepatocyte nuclear factor 3  
HSF- heat shock factor  
IGF-1- insulin-like growth factor I  
IL - interleukin  
ITF- intestinal trefoil factor  
LBD - ligand-binding domain  
LBD- ligand-binding domain

LEF-lymphoid enhancer binding factor  
MAPK - mitogen activated protein kinase  
mg - milligram  
Min-minute  
ml- milliliter  
mM-millimolar  
M-mole  
NFDM-non fat dry milk  
NFkB-nuclear factor kB  
PCR- polymerase chain reaction  
Pfabp - fatty acid binding protein promoter  
PG-R-prostaglandin receptor  
PKC- protein kinase C  
PSP-porcine spasmodic polypeptide  
RAR-1-retinoic acid receptor  $\alpha$  1 (RAR-1)  
RT- reverse transcription  
Sec-second  
SP- spasmodic polypeptide  
SRC - steroid receptor coactivator  
TBS - tris-buffered saline  
TCF-T cell factor  
TFF1-trefoil factor 1  
TNF - tumor necrosis factor  
TPA - tetradecanoylphorbol-13-acetate  
TTBS-tween 20 tris-buffered saline  
TXA2-R-thromboxane A2 receptor  
ul- microliter  
uM-micromolar  
USF- upstream stimulating factor

## 8. CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

## 9. PUBLICATIONS

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