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13. ABSTRACT ***(Maximum 200 Words)***

We studied the expression and regulation of the secreted FGF-BP protein that can enhance FGF activity and studied Its impact in vivo.
In human tissues we found that FGF-BP is significantly elevated in its expression in in vasive breast cancers (approximately 1/2 of invasive breast cancers) and rarely found expressed in normal breast tisue or in non-invasive *in situ* carcinoma. We report a significant, positive correlation of FGF-BP expression with the estrogen receptor status of the cancers.
We demonstrate transcription factors binding to the FGF-BP gene pomoter and show that members of the C/EBPbeta family are crucial transcriptional factors binding to the promoter. We show regulation by EGF of this binding.

We initated the generation of conditional, regulated transgene expression in mice due to the embryonically lethal phenotype observed with constitutively active promoters driving the transgene. As an alternative model, transient transgene expression in chicken embryos is initiated to assess to what extent embryonic toxicity can be monitored in this new model.

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INTRODUCTION

One mode of delivering active FGF from the extracellular storage site to its receptor is binding to a secreted carrier protein. The secreted FGF binding protein (FGF-BP) studied here can serve as such an an extracellular chaperone molecule for FGFs[1] (see Figure 1).

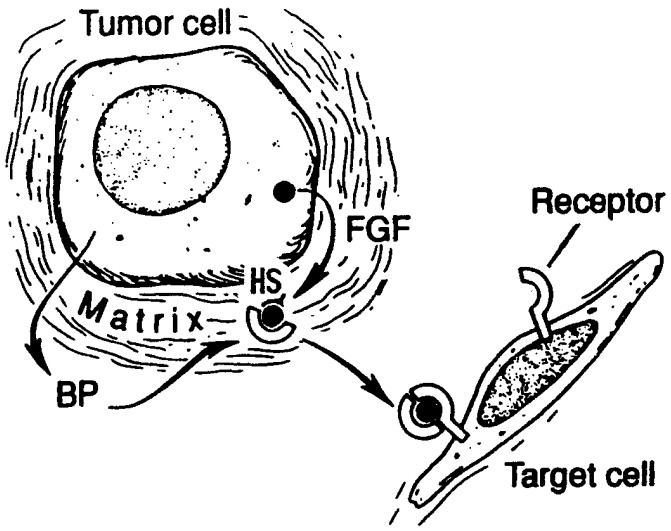


Figure 1. Model explaining the function of FGF-BP (BP)

Here we report on findings under:

Task 1. We had detected FGF-BP expression in a small series of invasive breast cancers and followed this here with a larger series which included information on clinico-pathological features.

Task 3. Transcriptional regulation of expression of FGF-BP in breast cancer cell lines was followed for cis-elements and trans-acting factors. Transcription factors in the C/EBPbeta family were analyzed for their impact on regulation of the FGF-BP promoter in more detail.

Task 4. We initiated the generation of conditional transgene expression in mice. We also generated a transient model of transgene expression in chicken embryos as a back-up.

BODY

TASK 1:
To assess the significance of FGF-BP expression for the clinical course of breast cancer using archival samples with known outcome and defined pathological features.

Work accomplished during the current award cycle:

Here we expanded our number of tumor samples (and controls) analyzed for FGF-BP mRNA expression using in situ hybridization described in the first cycle of this award. An expansion of the number of samples as well as staining for other markers and analysis of available data was carried out. The overview and statistical analysis of results obtained from the stainings of a series of samples is given in the Tables 1 and 2 below.

Table 1: Expression analysis for FGF-BP in human breast tissues.

FGF-BP	Negative	positive
Normal Breast	n = 24	4
In situ carcinoma	n = 26	8
Invasive cancer	n = 76	67

Statistical analysis of the data from Table 1 showed

1. a significant difference in the frequency of expression of FGF-BP amongst all groups ($p = 0.0008$; chi-square, Fisher's exact test);
2. a significant trend of increased frequency of expression from normal vs. in situ to invasive cancer ($p = 0.0002$ for trend; chi-square test).
3. a significant difference between normal and invasive cancer ($p = 0.0014$; chi-square, Fisher's exact test)
4. a significant difference between in situ carcinoma and invasive cancer ($p = 0.014$).
5. no significant difference between normal, non-malignant breast tissues and tissues with in situ carcinoma ($p = 0.36$; chi-square test).

These results from the expression studies of FGF-BP in breast cancer were next correlated with the available pathologic, clinical and molecular parameters associated with the samples.

1. Clinical and pathologic parameters were provided by CBCTR of NIH, who had the different samples characterized by expert pathologists from the NSABP (Dr. Soon Paik) and Baylor College of Medicine (Dr. Craig Allred).

2. Standard molecular parameters (ER, PR, HER-2) were generated by CBCTR and are provided by CBCTR.
3. Novel parameters that my laboratory studies, i.e. expression of the growth factor pleiotrophin (PTN) and its receptor anaplastic lymphoma kinase (ALK) [2-6]were generated at the same time as the stainings for FGF-BP and are used as positive controls.

(NOTE: For a few of the samples some of the parameters were not available, since some samples were lost from the tissue microarray during the staining or could not be evaluated.)

Table 2: Correlation of FGF-BP expression in invasive breast cancers with pathologic, clinical and molecular parameters.

Parameter	N	Spearman r (correlation)	p-value (two-tailed)	Significant (P<0.05)
T	113	-0.155	0.102	NS
N	96	-0.009	0.930	NS
M	119	-0.102	0.271	NS
# of LNodes	96	-0.019	0.854	NS
Stage	119	-0.083	0.372	NS
Grade	113	-0.116	0.222	NS
ER	116	0.205	0.027	Sig
PR	115	0.148	0.113	NS
HER-2	122	-0.126	0.167	NS
PTN*	126	0.610	<0.0001	Sig
ALK*	119	0.528	<0.0001	Sig
Overall Survival	119	0.085	0.357	NS

T, N, M is tumor size, nodal status (+/-) and existing distant metastasis; # of tumor positive lymph nodes, ER, PR (estrogen and progesterone receptor status) HER-2 overexpression of HER-2 growth factor receptor (immunohistochemistry).

*Refs.: [2-6]

Statistical analysis of the data was carried out using the Prism/Graphpad program. NS = not significant; sig = significant.

Methods:

Tissue samples.
Paraffin-embedded tissue samples were used for in situ hybridization and analyzed as described in the appendix materials. One set of tissue samples (n = 53) was from the Lombardi Cancer Center Tumor Repository. A second, independent set of samples (n = 152) was on tissue microarrays obtained through the NCI Cooperative Breast Cancer Tissue Resource (CBCTR). This tissue array contains samples from invasive breast cancer, in situ carcinoma as well as control breast tissues from reduction mammoplasty without malignancy (CBCTR Protocol # L-0060T to the P.I. of this grant, A.W.).

In situ Hybridization
The expression of FGF-BP mRNA in human breast tissue samples was assessed by in situ hybridization. The FGF-BP riboprobe consisted of a 668 bp internal sequence of FGF-BP cDNA [7], sub-cloned into the pRc/CMV vector (5.5 kb, Invitrogen). Digoxigenin-labeled antisense and sense riboprobes were made using the DIG RNA labeling kit (Roche) according to protocol. Tissue sections were cut (4 micrometers) and mounted on (+)-charged glass slides (Fisher Scientific; Pittsburgh, PA) using standard histology technique. In addition, tissue microarrays (TMAs) from the NCI Cooperative Breast Cancer Tissue Resource (CBCTR) were used. This TMA contained samples from invasive cancer as well as in situ carcinoma and control breast tissues from reduction mammoplasty.

Additional details and an overview of the method for *in situ* hybridization is described in the **appendix materials**.

TASK 1; INTERPRETATION OF THE DATA.

FGF-BP expression is detectable in close to 1/2 of breast cancer samples studied. The expression increases significantly from normal, non-malignant tissues and non-invasive in situ carcinoma to invasive cancers. However, there is no significant correlation of expression of FGF-BP in invasive cancers with overall survival of patients, tumor size or other clinico-pathological parameters (T, N, M, stage, grade). Only tumor size might have a potential negative correlation with FGF-BP expression ($r = -0.155$; $p = 0.102$), although the p-value is not significant (<0.05), but this could suggest that with increasing tumor size FGF-BP gets down-regulated.

With respect to standard molecular parameters used in routine analysis of breast cancers, a significant correlation between FGF-BP expression and ER was found although the correlation coefficient of 0.205 does not suggest a very strong correlation. This would suggest a reason why overall survival was not adversely correlated with FGF-BP since many of the ER-positive tumors have a good overall survival.

No significant correlation of FGF-BP expression with PR or HER-2 were found, although the correlation coefficient of 0.15 suggest a positive correlation for PR, but was not below the 0.05 level set for significance ($p = 0.11$). A positive correlation would be independently supportive of the notion of a positive correlation between active ER and FGF-BP expression.

Finally, a very strong correlation was apparent with respect to PTN and ALK [2-6] expression with correlation coefficients above 0.50 and p-values of <0.0001. This does suggest that the malignant transformation process affects FGF-BP and these latter parameters in parallel.

TASK 2:
To study regulators of the endogenous FGF-BP gene in breast cancer cell lines.

Work accomplished during current award cycle:
Here we followed up on the findings in earlier cycles that C/EBPbeta is a crucial transcriptional activator of FGF-BP in breast cancer cells (MDA-MB 468). We studied LIP and LAP isoform expression using Western blots (Fig. 1) and then studied the function by overexpression of the factors to assess (Fig. 2). These two proteins are the activating (LAP) and inhibitory or repressive (LIP) isoforms of C/EBPbeta.

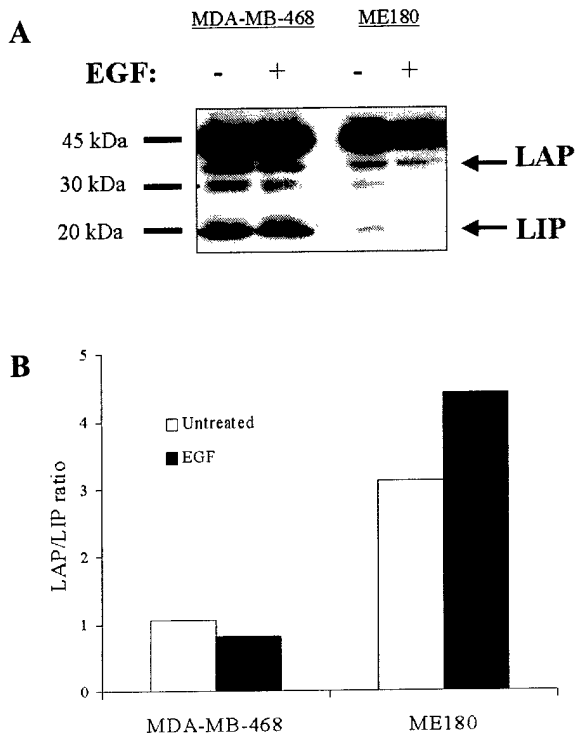
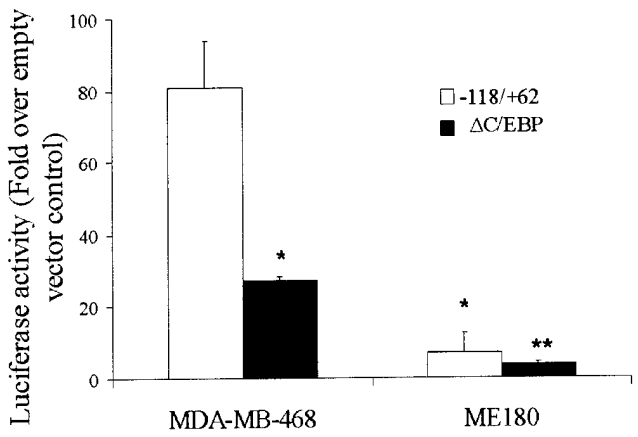
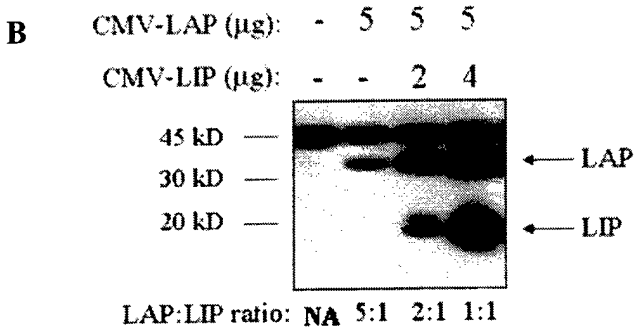


Figure 1. **Expression levels of endogenous C/EBPbeta in MDA-MB-468 breast cancer as compared to ME-180 squamous cell carcinoma cells.** *A*, A representative Western blot analysis of C/EBPb protein levels using 40 μg of nuclear extracts from untreated and EGF treated MDA-MB-468 and ME-180 cells. C/EBPb was specifically recognized using a polyclonal antibody specific for the C-terminus of the protein (C/EBPb (C-19)). The positions of LAP (35 kDa) and LIP (20 kDa) isoforms are indicated by *arrows*. *B*, Ratios of LAP to LIP in MDA-MB-468 and ME-180 cells. Levels of LAP and LIP were quantified from multiple exposures of Western blot analyses using densitometry, and corrected for levels of the 46 kDa nonspecific band. Values are expressed as actual ratios of LAP to LIP for each cell line.





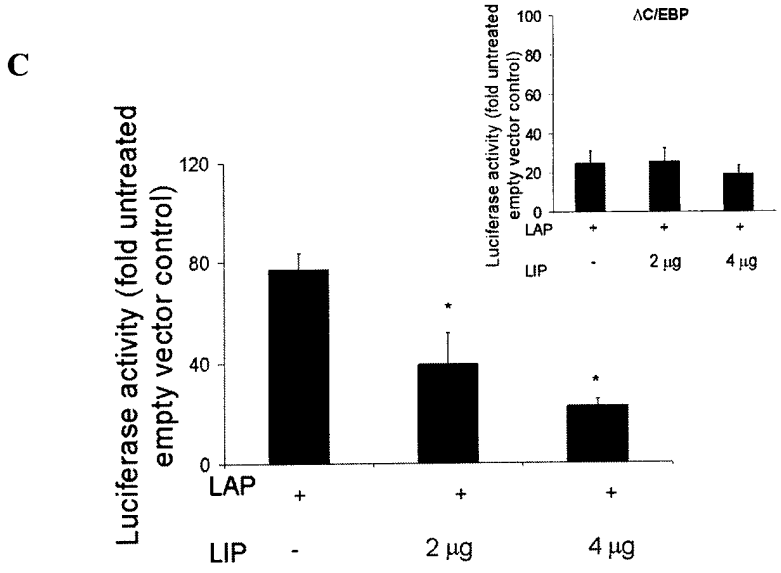


Figure 2. **Effects of LAP and LIP overexpression on the FGF-BP promoter activity (TEXT OF LEGEND ON THE NEXT PAGE).**

Figure 2 (graphs on previous page). **Effects of LAP and LIP overexpression on the FGF-BP promoter activity.** *A,* MDA-MB-468 cells and ME-180 were cotransfected with the indicated FGF-BP promoter constructs and 5 μg CMV-LAP. Relative luciferase activity of the cells transfected with ΔC/EBP (black bars) or -118/+62 (open bars) constructs are expressed as fold over control (empty vector transfected cell lines). *B,* Shown is a representative Western blot of C/EBPb protein levels in MDA-MB-468 cells transiently transfected with CMV-LIP and CMV-LAP. The levels of LAP and LIP were quantified from multiple exposures of Western blot analyses using densitometry, and corrected for levels of the 46 kDa band. Values are expressed as relative ratios of LAP to LIP for each transfection conditon. *C,* MDA-MB-468 cells were cotransfected with the -118/+62 or DC/EBP (*C, inset*) FGF-BP promoter constructs, 5 μg CMV-LAP, and the indicated amounts of CMV-LIP or empty vector (pcDNA3). MDA-MB-468 cells were treated were kept in serum free IMEM for 16 h. Values are expressed as the mean and S.E. of at least three experiments, each done in triplicate wells. Statistically significant differences relative to -118/+62 (*) or MDA-MB-468/DC/EBP (**) are indicated ($p<0.05$, t test).

TASK 3; Methods:

Plasmids- Human FGF-BP promoter fragments were cloned into the pXP1 promoterless luciferase reporter vector and have been described previously [8]. The MEK2 (K101A) dominant negative construct was provided by Dr. J. Holt (Vanderbilt University). The expression plasmids containing wild-type p38 (pCDNA3-Flag-p38), and constitutively active MKK6 (pCDNA3-Flag-MKK6(Glu)) were provided by Dr. R. Davis (University of Massachusetts). The expression vectors for human CEBPb-LAP and C/EBPb-LIP (CMV-LAP and CMV-LIP, respectively) were gifts from Dr. U. Schibler (University of Geneva) courtesy of Dr. J. Schwartz (University of Michigan). Wildtype C/EBPb mRNA contains three inframe AUG translation start sites, from which LAP and LIP are translated from the second and third sites, respectively [9]. The second in frame AUG is flanked by an imperfect Kozak's sequence, GACCATGG [10], compared to the Kozak's consensus sequence of CCA/GCCAUGG [11,12], whereas the third inframe AUG is flanked by a perfect Kozak's sequence [9], resulting in translation of both LAP and LIP. The CMV-LAP construct contains only the second and third translation start sites, but both are flanked by perfectly matched Kozak's sequences resulting in the more efficient translation of LAP alone [9]. The effects of dominant negatives or activated constructs were compared to their empty vector control or with the empty vector pCDNA3 (Invitrogen).

Transient Transfections and Reporter Gene Assays- Twenty-four hours before transfection MDA-MB-468 cells were plated at a density of $3x10^6$ cells in 10-cm dishes. pRL-CMV *Renilla* luciferase reporter vector (Promega; Madison, WI) was included as a control for transfection efficiency. MDA-MB-468 cells were transfected by electroporation as described by Raja *et al.* [13]. Briefly, cells were trypsinized and washed twice by centrifugation in IMEM containing 10% FBS. The cells from each plate were then resuspended in 400 μl IMEM containing 20% FBS. A total of 30 μg plasmid DNA (29 μg of FGF-BP promoter construct, 3.0 ng of pRL-CMV) was added to the cell suspension 5 minutes before electroporation. For co-transfection, 24 μg of -118/+62Luc FGF-BP promoter construct, 5 μg or indicated amounts of expression vector, and 3.0 ng of pRL-CMV were added to cells. Electroporation of the entire cell sample was carried out in a cuvette with an electrode gap of 0.4 cm at 350 V and 500 mF, using a BioRad GenePulser II (Bio-Rad; Hercules, CA). The electroporated cells were then distributed equally to a 6-well plate, each well having been pre-filled with 3 ml of IMEM with 10% FBS. Cells were allowed to recover and attach for 16 hours before treatment. Transfected cells were washed twice with serum-free IMEM, treated with or without EGF (10 ng/ml) in serum-free IMEM for 16 hours, and then lysed in 150 μl of passive lysis buffer (Promega). 20 μl of extract was assayed for both firefly and *Renilla* luciferase activity using the Dual-Luciferase™ reporter assay system (Promega). To correct for transfection efficiency, and a small background induction (1.5-2.0 fold) of the pRL-CMV plasmid by EGF [14], *Renilla* luciferase values were corrected for protein content, and these numbers were then used to normalize firefly luciferase values. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

Western Analysis- 40 μg of crude nuclear extracts from untreated or EGF-treated MDA-MB-468 and ME-180 cells, or 20 μg of lysates from MDA-MB-468 transiently transfected cells were electrophoresed on Novex® precast 4-20% tris-glycine polyacrylamide gels (Invitrogen) at 150 V for 80 minutes. The protein was then transferred to polyvinylidene difluoride membranes (Millipore) for 2 hours at 200 mA. Blots were blocked for 1 hour in PBST (1x phosphate buffered saline, 0.5% Tween 20) containing 4% bovine serum albumin (Sigma; St. Louis, MO), and then incubated for 1 hour in 1x PBST/ 0.4% bovine serum albumin, containing antibodies (1 μg/ml) for C/EBPb (C-19) (Santa Cruz). Blots were washed with PBST (without bovine serum albumin) four times for 5 minutes each, with agitation. Blots were then incubated for 1 hour in antibody solution containing a 1:5000 dilution of horseradish peroxidase labeled donkey anti-rabbit immunoglobulin (Amersham) and washed as before. Lastly, blots were assayed for enhanced chemiluminescence using SuperSignal® West Pico Chemiluminescent Substrate (Pierce) and enhanced chemiluminescence film (Hyperfilm ECL; Amersham). Signal intensities of the 46, 36 (C/EBPb-LAP), and 20 kilodalton (C/EBPb-LIP) bands were measured by densitometry using multiple exposures to Hyperfilm ECL to assess the linear range. Expression of LAP and LIP were corrected for loading differences by comparing to the band intensity of the 46 kDa band, as described by Zahnow *et al.* [15].

Statistics – The GraphPad/Prism software package was used for graphics and data evaluation. ANOVA was applied for continuous variables and chi-square (Fisher's exact test) for discontinuous variables. p. values < 0.05 were considered significant.

TASK 3; INTERPRETATION OF THE DATA

Earlier mutational analysis had revealed that the AP-1 and C/EBP sites on the FGF-BP gene promoter were required for the EGF effect, whereas deletion of the C/EBP site resulted in a significant increase in promoter basal activity indicating a basal repressive control mechanism. This was corroborated by gel shift analysis showing binding of transcription factors to the C/EBP site.

We now report that MDA-MB-468 cells express high endogenous levels of both the activating (LAP) and inhibitory (LIP) isoforms of C/EBPb (=C/EBPbeta) (Figure 1). Overexpression of C/EBPb-LAP in MDA-MB-468 cells resulted in a large 80-fold increase in FGF-BP promoter basal activity (Figure 2). This activation was reversed by coexpression of LIP (Figure 2). Thus the balance of these two proteins is the deciding factor in the activity of C/EBPbeta.

TASK 4:
Transgenic FGF-BP expression.

We had found that expression of hFGF-BP under the control of CMV, K14 or MMTV is embryonically lethal. Thus we initiated two series of studies to circumvent this:

1. **Conditional (Tetracycline regulated) FGF-BP transgene expression in mice**
2. **Transient expression model in chicken embryos**

Work accomplished during the current award cycle:

1. **Conditional (Tetracycline regulated) FGF-BP transgene expression in mice**

To bypass embryonic lethality of FGF-BP expression in mice and study the effect of BP transgene expression in mice, we initiated the generation of mice with tetracycline-regulatable BP expression.

At first we generate a vector that could be used as a transgene: tetracycline-response element containing FGF-BP expression vector (= tetRE-FGF-BP). This vector was based on our earlier work on conditional expression of FGF-BP in cell lines under tetracycline control [16].

As a first set of animals we generated mice containing FGF-BP under the control of a tetracyline response element (tetRE-FGF-BP). These mice still showed some lethality due to leakage of the tetRE promoter that was also found in cell studies. Still, we were able to generate two distinct stable transgenic lines that contained the tetRE-FGF-BP as an integrant.

It is planned that these tetRE-FGF-BP mice will then be crossed with a second set of animals that express the tetracycline-regulatable transactivator (tTA). Depending on the promoter (K14 or MMTV or CMV) used to express the tetracycline-transactivation protein (= tetRE tTA), FGF-BP will then be regulatable in different tissues in the resulting bitransgenic animals by administration of doxycycline (a tetracycline derivative) and the resulting phenotypic effects can be studied. The tTA expressing mice are available from a number of laboratories and vendors.

2. Transient expression of FGF-BP in chicken embryos

(Comment:
Although this is beyond the scope of this proposal, the following series of experiments was initiated due to the embryonically lethal phenotype generated by FGF-BP expression in mice and thus will be documented and commented on here.)

Chicken embryo studies have been used for many decades in biology. We have employed this model to study angiogenesis factors on the chorio-allantois membrane (CAM) of the embryos. More recent studies published e.g. in Cell (Li et al 1995) have demonstrated in this model that application of FGF can induce limb bud formation in chick embryos and have analyzed the signaling pathways for that. We set up a particular version of the chicken embryo as a model system which allows continuous microscopic monitoring of phenotypic effects in the live embryo. For this model, day 3-fertilized eggs are cracked and the contents placed on 10 ml of DMEM in 10 cm petri dishes (see Fig. 1A,B). In a humidified incubator at 39°C a high portion (>2/3) of the chicken embryos will grow on top of the yolk for at least another week and approximately 1/3 to 1/4 till day 12. Occasionally the embryos will even grow to full maturity if the experiment is allowed to continue and we have had viable, fully-matured chicks grown from this approach. Genes can be expressed for >4days after a single plasmid injection into the yolk sac (see Fig. 1C,D).

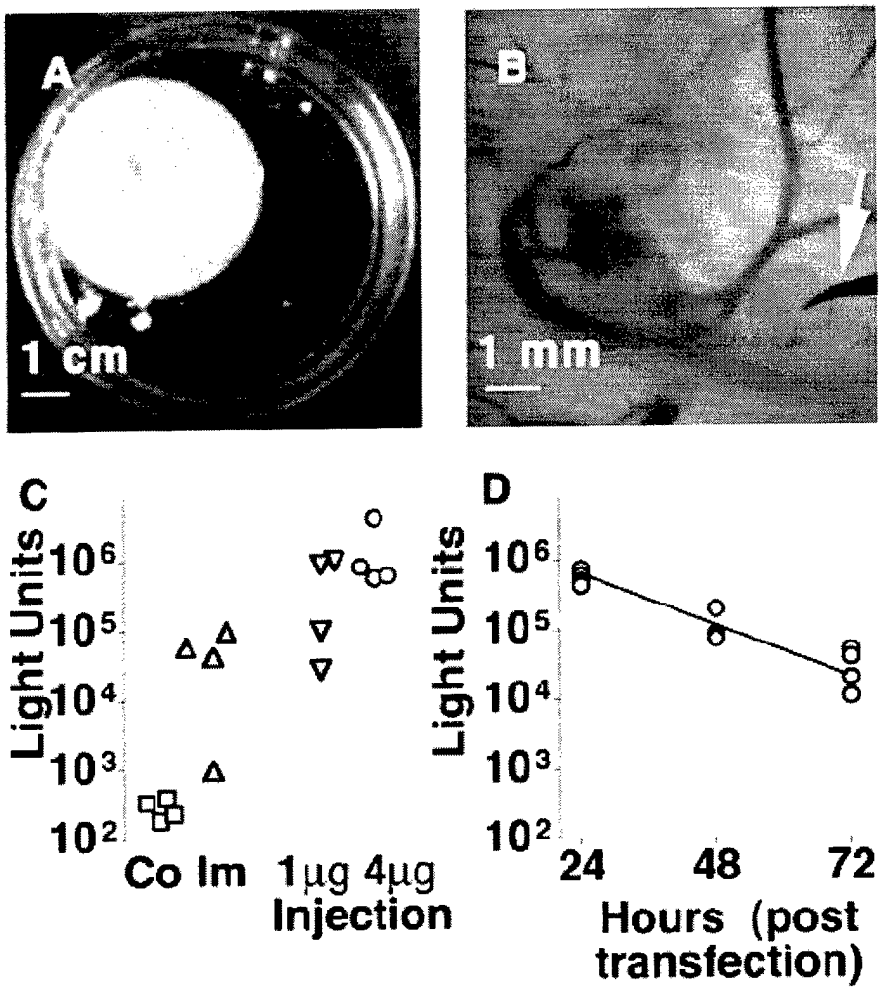


Figure 1. Gene Expression in the Chicken Embryos.

(A, B) Overview of the model. Different magnification of day 4 chicken embryos that were used for microinjection. The arrow in panel B points to the injection needle (black). **(C)** Embryos were microinjected with 25 ml of lipofectamine without (Control) or with 1 mg of CMV luciferase plasmid around the body of the embryo (Im = DNA immersion) or with 1 or 4 ug of the plasmid into the allantoic sac. Luciferase activity was measured 24 hours after microinjection. **(D)** Time course of luciferase activity after 4 ug of CMV luciferase injection into the allantoic sac. Each data point represents measurements from an individual embryo. Note the logarithmic scale of the Y-axis.

TASK 4; Methods:

In Vitro Cultivation of Embryos. Three day old fertilized chicken (Gallus gallus) eggs (CBT Farms; Chestertown, Md.) were opened and intact embryos with yolk were placed in 10 x 2 cm polystyrene culture dishes (Corning; Corning, New York). Embryos were maintained in a humidified water-jacketed incubator at 39°C prior to and following transfection. The human FGF-BP (hFGF-BP), green fluorescent protein (GFP), luciferase and β-galactosidase expression vectors were constructed employing the pRc/CMV vector (Invitrogen, San Diego, CA). The hFGF-BP plasmid contained the hFGF-BP open reading frame and has been described previously[1]. Various concentrations of plasmid constructs were incubated with 25 ml Lipofectamine™ reagent (Life Technologies) at room temperature for 45 min. 25 μl of the liposomal mixture were injected into each embryo using a Hamilton syringe (Hamilton; Reno, NV) under 3X magnification with an Olympus SZH10 steromicroscope.

To quantitate gene transfer, CMV luciferase was used as a vector and embryos were harvested and homogenized in 150 μl Passive Lysis Buffer (Promega) at various time points following transfection. The Luciferase Reporter assay system (Promega) was used to detect firefly luciferase activity in 100 μl homogenate utilizing a Monolight 2010 luminometer.

TASK 4; INTERPRETATION OF THE DATA

- We have now generated transgenic animals that contain a tetracycline-regulatable FGF-BP construct.
- We established a method to express transgenes as plasmid expression vectors transiently in chicken embryos.

KEY RESEARCH ACCOMPLISHMENTS

1. Significant positive correlation of expression of FGF-BP in invasive breast cancer samples with ER as well as the growth factor pleiotrophin and receptor anaplastic lymphoma kinase.
2. Analysis of C/EBPbeta isoforms driving FGF-BP promoter activity in breast cancer cells.
3. Initiation of tetracycline-regulatable transgene expression of FGF-BP in mice.
4. Establishing an alternative transient transgenic expression system for FGF-BP in chicken embryos.

REPORTABLE OUTCOMES

Posting for the NCI/NIH website on in situ hybridization (draft):

Henke et al.; CBCTR website NCI/NIH

CONCLUSIONS

- FGF-BP is highly expressed in approximately 1/2 of invasive breast cancers, correlated with ER and the PTN/ALK growth factor pathway and rarely expressed in normal breast tissues or in in situ carcinoma.

- The FGF-BP gene is regulated at the transcriptional level and utilizes the transcription factor family C/EBPbeta as a major mechanism that involves interaction between activating and inhibitory isoforms of transcription factors.

- FGF-BP expression as a regulatable transgene in mice is feasible.

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APPENDICES

Henke et al.; CBCTR website NCI/NIH

(UNDER REVIEW FOR THE CBCTR WEBSITE AT NIH/NCI)

IN SITU HYBRIDIZATION WITH DIGOXIGENIN LABELED RNA PROBES

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Introduction

When investigating the RNA expression in tissues one of the main concerns is to distinguish between expression levels in particular cell types and structures. When using northern blot or real time PCR with heterogeneous tissue samples it is often difficult to reach exact conclusions. The in situ hybridization (ISH) is established as a method allowing demonstration of the RNA expression by using specific probes without loosing the morphological information since the tissues are preserved in there structure.

The protocol described here uses Digoxigenin labeled RNA-Probes thus avoiding any radioactive isotopes and was used in our lab for more than 1000 slides including more than 100 tissue micro arrays. Positive staining results will appear as a violet to brown staining in individual cells, mainly in the cytoplasm. Normally unstained nuclei can be observed surrounded by stained cytoplasm (**Figure 1a**). Unspecific staining can be expected especially in collagen structures and usually appears blue rather than violet. Counterstaining with e.g. Haematoxilin is not necessary and not recommended since the low staining intensity of the ISH may otherwise be difficult to evaluate. Tissue structures can still be determined even in completely negative tissue (**Figure 1b**).

When staining tissue micro arrays we recommend establishing the method of ISH and the quality of each individual probe set (antisense and sense) first on an appropriate number of full sections of according pathological and reference tissues (e.g. for a breast cancer array: two sets of 10 invasive breast cancers and 10 normal breast samples, stained in the same batch. One set used for antisense and one for sense). This is especially important since due to their limited availability and high value usually only one tissue micro array will be stained with the antisense probe and no second array for the sense probe. When advancing to tissue micro arrays with established probes we also recommend to include several slides of previously tested tissues in the same batch with the array that are known to be positive or negative for the RNA of interest. Two sets of these should be stained with the antisense and sense probe. This provides appropriate external positive and negative controls for the array.

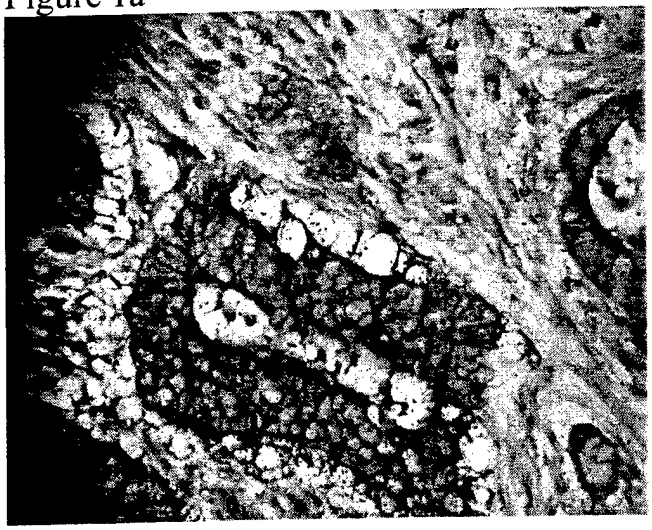
When evaluating the results of different batches of ISH high variation of staining intensity may be observed. When using tissue micro arrays each of them should be evaluated individually by first identifying the highest and lowest staining intensity on the slide.

To evaluate results we suggest using a relative scale for each array defining the highest observed staining as "+++" and the lowest as "-". Recommended coding to evaluate staining results of the individual cores is shown in **table 1**.

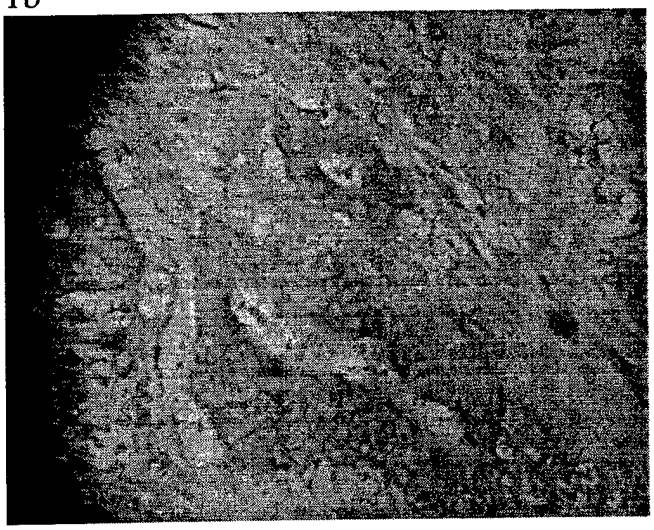
Table 1

Negative staining	-	no staining observed in this core
	+/-	slight signals but no certain positivity
Positive staining	+	certain positive staining in at least some cells
	++	medium staining in all cells or light staining in some cells and high staining in others
	+++	high staining in all cells and/or very high staining in at least 20% of the cells
N/A	x	core missing or the tissue of this core is necrotic and the RNA is degraded
	-NT	the appropriate cells (i.e. in breast arrays: cancer cells or breast epithelial cells) are not present in this core.

Figure 1a



1b



Representative staining results

1a) Colon Cancer stained with antisense RNA-probe. (400x)

1b) Sequential section stained with the according sense RNA-probe (negative control)

1. Solutions

SOLUTION (volumes are recommendations for 3-4 ISH)	NEEDED FOR
1) Digoxigenin labeled RNA-Probe / approx. 600 ng per slide (microarray)	
2) Xylene (RNA-grade)	Day 1
3) Ethanol 100% (RNA-grade)	Day 1
4) DEPC-H_2O (Diethyl Pyrocarbonate) / 8 liters for preparation of solutions - add 1ml DEPC per 1l H_2O, stir o/n then autoclave next morning	Day 1 and as stock
5) PBS 1x / 1 liter - dilute from 10x Stock with DEPC-H_2O	Day 1
6) PBS 10x / 500 ml - commercial or: - 80g NaCl, 2g KCl, 11.5g Na_2HPO_4*$7H_2O$ in 500 ml DEPC-H_2O	as stock
7) Proteinase K in PBS 1x / 200 ml per ISH - add on Day 1: 2 mg Proteinase K to 200ml PBS (final conc. 10 µg / ml)	prepare fresh on Day 1
8) SSC 2x / 1 liter - dilute from SSC 20x with DEPC-H_2O	Day 1
9) SSC 20x / 1 liter - commercial or: - 3M NaCl, 0,3 M Na-citrate, ad 1l DEPC-H_2O	as stock
10) 0,2M HCl / 1 liter - 1 Part 6N HCl, 29 Parts DEPC-H_2O	Day 1
11) TEA-HCl (0,1M Triethanolamine-HCl at ph 8,0) / 2 liters - dissolve 18,6 g TEA-HCl (RNA-grade) in 900 ml DEPC-H_2O - titrate to pH 8,0 with 10N NaOH (takes time, solution is a buffer!) - ad 1l DEPC-H_2O	Day 1
12) 0,25% Acetic anhydride in TEA-HCl / 200 ml per ISH - add on Day 1: 500 µl Acetic anhydride in 500ml in 200 ml TEA-HCl	prepare fresh on Day 1
13) Hybridization Solution / 200 µl (small sample) – 400 µl (tissue array) per slide - Sigma®, catalog number H7782 (calculate 200-400 µl/Tissue)	Day 1
14) dd-H_2O / several liters to prepare solutions and approx. 2 liters / ISH	Day 2 + Day 3
15) STE Buffer / 1 liter - 500 mM NaCl, 20 mM Tris-HCl pH 7,5, 1mM EDTA in dd-H_2O	Day 2
16) RNAse A in STE Buffer / 200 ml per ISH - ad 2g of RNAse to 200ml STE Buffer	prepare fresh on Day 2

17) SSC 2x / 2 liters - 200 ml SSC 20x, ad 2 liters dd-H_2O	Day 2
18) SSC 2x + Formamide (1:1) / 200 ml per ISH - 100 ml Formamide, 20 ml SSC 20x, 80 ml dd-H_2O	prepare fresh on Day 2
19) SSC 1x / 200 ml per ISH - 10 ml SSC 20x, 190 ml dd-H_2O	Day 2
20) SSC 0,5x / 200 ml per ISH - 5 ml SSC 20x, 195 ml dd-H_2O	Day 2
21) Buffer #1 / 3 liters - 100 mM Tris-HCl pH 7,5, 150 mM NaCl (8,77 g/l) in dd-H_2O - FILTER	Day 2 + Day 3
22) 2% Horse Serum in Buffer #1 - add 4 ml of Horse Serum to 200 ml Buffer #1	prepare fresh on Day 2
23) Anti-Digoxigenin antibody solution - dilute in Buffer #1: (calculate 500-700 µl / slide) 1:250 anti-DIG-FAB-AB-Fragments, 1:100 Horse Serum	prepare fresh on Day 2
24) Buffer #2 / 3 liters - 100 mM Tris-HCl pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$ in dd-H_2O - FILTER	Day 3
25) NBT/BCIP Substrate Solution (**Light sensitive!** Wrap in aluminum-foil) - dilute in Buffer #2: (calculate 500 – 1000 µl / Tissue) 33,75µl / 10ml (3,75mg / 10ml) of NBT (nitroblue tetrazolium) 35µl / 10ml (1,75mg / 10ml) of BCIP (toluidinum salt)	prepare fresh on Day 3
26) Buffer #3 / 2 liters - 10mM Tris-HCl pH 8,0, 1mM EDTA in dd-H_2O - FILTER	Day 3
27) 0,5% Tween 20 in dd-H_2O / 200 ml per ISH	prepare fresh on Day 3
28) Sealing solution for microscopical slides	Stock

2: Material / Work environment

1) RNAse ZAP® Spray	
2) 20+ Plastic containers ("buckets") to hold the solutions during the ISH. Container-volume 200 ml	
3) Plastic slide holders for 24 slides	
4) Plastic trays ("slide chamber") to hold slides vertically during special steps like the o/n hybridization, the o/n antibody incubation and the NBT/BCIP staining	
5) Kimwipes®	
6) Parafilm®	
7) Plastic – Pasteur pipettes	
8) Vacuum system for at least 500 – 1000 ml of volume	
9) Empty drawer to develop during NBT/BCIP staining	
10) Workbench treated with RNAse ZAP®	
11) Sterile 1 liter vacuum filter system to prepare the solutions	
12) Ice on day one	
13) Water bath at 37°C	
14) Oven at 55°C and 65°C (do not use an incubator for cells or bacteria)	
15) Incubator at 42°C (do not use an incubator for cells or bacteria)	
16) Cover slips for microscopical slides	

3. Procedure

3.1. Preparations before the actual ISH

3.1.1. General considerations

- GLOVES MUST ALLWAYS BE WORN when handling the slides, any of the solutions or materials for day one or when handling the RNA-probes.
- Clean the workbench and all bottles, beakers and graded cylinders for solutions for day one with RNAse ZAP® and let them completely dry before proceeding further. Cover them with aluminum foil while drying.
- Whenever possible use sterile 15 ml or 50 ml Sarstedt® or Falcon® tubes for measuring and handling small volumes of solutions for day one. They can be considered RNAse free. Use them especially to prepare and store the hybridization solution with your RNA probe on day one.

3.1.2. Before Day 0

- Make at least 4 l of DEPC-H_2O (solution 4). If you prepare all solutions for the first time: 8 l.
- Clean the workbench with RNAse ZAP®
- Prepare solutions 5), 6), 8), 9), 10) and 11) with DEPC-H_2O
- Prepare solutions 15), 17), 21), 24) and 26) with dd-H_2O
- The three Buffer solutions must, all the others should be filtered (sterile1 liter vacuum filter system). Filter DEPC based solutions first if you want to use one filter for all. Wash filter between solutions for day one with DEPC-H_2O, later between solutions for day two and three with dd-H_2O

3.1.3. Day 0

- DO NOT MICROWAVE SLIDES, DO NOT KEEP SLIDES AT 55°C OVER NIGHT. Some other protocols recommend this preparation to remove excess paraffin. However this treatment reduces the RNA quality
- Clean the workbench (again), the plastic containers ("buckets") for day one and the slide holder with RNAse ZAP®. Place buckets and slide holder on and cover with aluminum foil. Let dry over night

3.1.4. Day 1

- Place slides in slide holder. Incubate slides in holder for 1 hour at 55°C
- Increase Oven to 65°C one hour before the first Xylene step and incubate slides for one more hour at 65°C
- Fill all **buckets for Day 1**:
  - Xylene I (Chemical hood!)
  - Xylene II (Chemical hood!)
  - Ethanol 100% I
  - Ethanol 100% II
  - DEPC-H_2O
  - PBS 1x
  - PBS 1x (later add: Proteinase K)
  - SSC 2x (DEPC-H_2O)
  - 0,2 M HCl
  - 0,1 M TEA-HCl pH 8,0
  - 0,5 ml acetic anhydride in 200 ml 0,1 M TEA-HCl pH 8,0
- Place the two PBS buckets in a 37°C water-bath
- Calculate RNA-probes and hybridization solution:
  a) needed volume of hybridization solution:
  200-400 µl/Tissue (200µl for small e.g. Cell pellets, 400 µl for Arrays)
  b) needed volume for antisense (and sense) RNA-probe:
  RNA-amount: The final RNA-concentration in the hybridization solution should be 1,5 µg RNA / 1,0 µl solution.

Example:
for 5 tissue array slides calculate 2000 µl hybridization solution and 3000 µg AS-RNA-Probe

3.1.5. Day 2

- ☐ Fill all **buckets for Day 2:**
  - SSC 2x I (dd-H_2O)
  - SSC 2x II (dd-H_2O)
  - STE Buffer
  - STE Buffer (add later: RNAse A)
  - SSC 2x + Formamide (1:1)
  - SSC 1x
  - SSC 0,5x
  - Buffer #1
  - Buffer #1 + 2% Horse serum (add later)
- ☐ Place the STE buffer buckets in 37°C water bath
- ☐ Place the SSC 2x + Formamide, the SSC 1x and the SSC 0,5x buckets in 42°C Incubator
- ☐ Take Horse Serum out of the Freezer (DO NOT heat over 37°C or microwave for thawing)

3.1.6. Day 3

- ☐ Fill all **buckets for Day 3:**
  - Buffer #1 I
  - Buffer #1 II
  - Buffer #2 I
  - Buffer #2 II
  - Buffer #3
  - 0,5% Tween
  - dd-H_2O

3.2. Day 1 – Protocol

3.2.1. De-Paraffination

- ❑ Pick up the slide holder from the 65°C oven (reset temperature to 55°C)
- ❑ Place the slide holder in Xylene I (Chemical hood!) ..... 5 min
- ❑ Xylene II (Chemical hood!) ..... 10 min
- ❑ Ethanol 100% I ..... 5 min
- ❑ Ethanol 100% II ..... 5 min
- ❑ DEPC-H_2O ..... 5 min

3.2.2. Protein-Digestion

- ❑ PBS I (37°C) ..... 5 min
- ❑ Add Proteinase K to PBS II (2 mg) while slides are in PBS I
- ❑ PBS II + Proteinase K (37°C) ..... 10 min
- ❑ PBS I (37°C) again ..... 5 min
- ❑ DEPC-H_2O (from here room temperature again) ..... 5 min

3.2.3. De-Proteinization

- ❑ 0,2 M HCl ..... 15 min
- ❑ While in HCl: add acetic anhydride to 0,1M TEA-HCl pH 8,0 bucket II
- ❑ While in HCl: pick up hybridization-solution from –20°C and RNA-probe(s) from –80°C PLACE BOTH ON ICE!
- ❑ While in HCl: prepare slide-chamber: double layer of wet filter paper, with Parafilm® on top

3.2.4. Acetylation

- ❑ 0,1M TEA-HCl pH 8,0 I ..... 5 min
- ❑ 0,1M TEA-HCl pH 8,0 II + 0,25% acetic anhydride ..... 15 min
- ❑ While in (TEA II + acet. anhy.): Mix needed volume hybridization solution with RNA-Probe. Keep ready solution on ice. Refreeze the remaining RNA-probe(s) at –80°C.

3.2.5. Hybridization

- ❑ SSC 2x (DEPC-H_2O) ..... 5 min
- ❑ Take out one slide at a time. Then do for each slide:
  - o Dry backside and front areas without tissue carefully with a Kimwipe®
  - o Place the slide vertically in the slide chamber
  - o Pipette the hybridization solution / RNA-probe mix on the tissue
- ❑ After the last Slide: seal the slide chamber with Parafilm® and place **O/N in 42°C Incubator**

3.3. Day 2 – Protocol

3.3.1. Post-Hybridization wash

- ❑ Pipette 1,0-2,0 ml of SSC 2x on each slide to dilute the hybridization solution. Use a plastic Pasteur pipette
- ❑ Suck tissues CAREFULLY almost dry with vacuum system (1 ml pipette-tip on the vacuum tube). Process one slide at a time. Do not allow the tissue to dry out completely
- ❑ Pipette 1,0-2,0 ml SSC 2x back on the tissue directly after the vacuum step
- ❑ After one complete round restart again to vacuum one slide at a time.
- ❑ Pick up the slide directly after the second vacuum step and dip it shortly in the SSC 2x I bucket. Then place it in an empty slide holder in the SSC 2x II bucket where you collect the slides
- ❑ When all slides are processed and collected in the SSC 2x II bucket, wait ..... 5 min

3.3.2. RNA-Digestion

- ❑ STE Buffer I (37°C) ..... 5 min
- ❑ While in STE Buffer I: Add 2mg RNAse A to STE Buffer II
- ❑ STE Buffer II + RNAse (37°C) ..... 10 min
- ❑ STE Buffer I (37°C) again ..... 5 min

3.3.3. Re-Fixation

- ❑ SSC 2x + Formamide (1:1) (42°C) ..... 10 min
- ❑ SSC 1x (42°C) ..... 5 min
- ❑ SSC 0,5x (42°C) 5min

3.3.4. Blocking

- ❑ Buffer #1 ..... 1 min
- ❑ Buffer #1 + 2% Horse Serum ..... 30 min
- ❑ While in Buffer #1 + HS: Prepare Antibody solution (Buffer #1 + AB + 1% Horse Serum)

3.3.5. DIG-Antibody-solution Application

- ❑ Take slides out of the blocking solution one after at a time and:
  - o Dry backside and front areas without tissue with a Kimwipe®
  - o Place slide in slide chamber
  - o Pipette Antibody solution (500+ µl / slide)
- ❑ After the last slide: seal the chamber with Parafilm®
- ❑ Place chamber in **4°C Fridge** ..... **overnight**

3.4. Day 3 – Protocol

3.4.1. Antibody washout

- Leave slides in the chamber and pipette 1000-2000µl Buffer #1 on each tissue (plastic pipette)
- After „full round" carefully vacuum liquid away and DIREKTLY re-pipette some Buffer #1
- Do a second round of vacuum. Each time a slide is finished dip it in Buffer #1 I and the place it in a slide holder in Buffer #1 II
- Once all are collected in the holder in Buffer #1 II: transfer the slides in the holder to Buffer #2 I
- Buffer #2 I ..... 5 min
- Buffer #2 II ..... 5 min
- While in Buffer #2: Prepare staining solution (NBCI/NBT in Buffer #2) (prepare in a Falcon® or Sarstedt® tube wrapped in aluminum foil)

3.4.2. Staining

- Take one slide at a time out of the Buffer #2 II, then:
  - Dry backside and front areas without tissue with a Kimwipe®
  - Place slide in the slide chamber
  - Pipette staining solution on tissues with a plastic pasteur pipette. *Note: Speed is crucial if multiple slides are processed since the first slides may already be fully stained before the last one is processed.*
- When all slides are in the chamber: Place it carefully in a drawer to develop in the dark
- Check every 10 min for staining process. If uncertain, take one of the first slides to the microscope to check and re-pipette staining solution afterwards if necessary

3.4.3. Stopping staining and cleaning

- When stained enough (usually after 20-30 min.), pick up the slides one at a time in the order they were processed, dip them in Buffer #2 and place them in a slide holder in Buffer #3
- When all slides are processed and in Buffer #3: ..... wait 5 min
- 0,5% Tween 20 (move bucket carefully to wash slides) ..... 5 min
- dd-H_2O: Place rack in bucket, go to the sink and wash under the dd-H_2O tap (slow flow) ...2 min
- Take slides out, place vertically on filter paper and let them COMPLETELY dry . at least 20 min
- Apply sealing solution on slides and cover with slip (Chemical hood)
- Wait at least 24hrs before storing slides upright in a box!

4. Further reference

Panoskaltis-Mortari, A. and R. P. Bucy (1995). "In situ hybridization with digoxigenin labeled RNA Probes: facts and artifacts." Biotechniques 18(2): 300-7