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Abstract

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KNOCKDOWN OF FANCL MAY IMPAIR MULTI-LINEAGE PROGENITOR EXPANSION FROM CORD BLOOD STEM CELLS.

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Abstract

Fanconi anemia (FA) is a rare, inherited chromosomal instability disorder characterized by bone marrow failure and susceptibility to cancer. Cells from patients with FA accumulate excessive chromosome breaks when exposed to DNA damaging agents; therefore, providing evidence that the FA pathway functionally exists as an integral part of DNA repair. Recent evidence suggests that alternative functions of the FA pathway exist, yet precisely for what cell type and context is unknown. Our laboratory has evidence that the FA pathway may regulate the signaling output of Wnt/β-catenin, a known signal transduction pathway that regulates a hematopoietic stem cell’s ability to expand and self-replicate. Specifically, FANCL may enhance the signaling output of β-catenin. Given the relationship between FANCL and this pathway, as well as the observation that hematopoietic stem cells from FA patients are remarkably susceptible to apoptosis and display defective stem cell properties, we investigated whether introducing the FA phenotype in cord blood stem cells impacts their self-renewal properties in colony forming cell assays. We tested a set of small hairpin RNA constructs for their ability to knockdown FANCL by western blot and qRT-PCR analysis. Our preliminary data shows that FANCL-deficient hematopoietic stem cells have reduced capacity to form multi-lineage progenitors. Based on this preliminary work, we will further investigate if perturbation of the Wnt/β-catenin pathway is the mechanistic explanation for why these cells have impaired multi-lineage potential. Identifying these mechanisms may provide insights into how Wnt/β-
Catenin signaling may be modulated by other pathways to maintain the hematopoietic stem cell pool. Thus, inherited or acquired perturbations of this regulation may lead to bone marrow failure.

**Introduction**

Fanconi anemia (FA) is a rare, inherited chromosome instability disorder characterized by bone marrow failure and cancer predisposition (Taniguchi and D’Andrea, 2006). The most notable symptom of FA, bone marrow failure or aplastic anemia, generally presents within the first decade of life; the primary cause of mortality in FA patients is aplastic anemia (Taniguchi and D’Andrea, 2006). In addition, it is observed that FA patients have a 100-fold higher chance of developing acute myeloid leukemia (AML) (Muller and Williams, 2009). It is estimated that up to 52% of FA patients will develop AML (Berwick et al., 2010). The hallmark feature of FA is excessive chromosomal breaks in the cells of FA patients when those cells are treated with DNA damaging agents such as mitomycin C, compared to cells from a healthy individual, whose cells will repair the damage (Tulpule et al., 2010). This suggests that the FA syndrome is manifested from mutations within a DNA repair mechanism. Although rare, the FA disorder is a model disease to study because it provides novel insight into a variety of important biological processes concerning disease etiology, such as DNA repair mechanisms and cancer transformation.

The FA phenotype originates from a biallelic, autosomal mutation of any of the fifteen genes in the Fanconi pathway (excluding FANCB, which is a biallelic X-linked mutation) (Taniguchi and D’Andrea, 2006). The FA pathway is comprised of 15 known proteins (subtypes FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N), which are thought to exist
in a large, multisubunit complex as part of a DNA damage response (Tulpule et al., 2010). The majority of these (FANCA, B, C, E, F, G, L, M) form what is called the FA core complex (Tulpule et al., 2010). The core complex has two known functions, the first being recruitment of the FA proteins to the site of DNA damage and the second as an E3 ubiquitin ligase. The FANCL protein, however, is the only protein of the core complex that has ligase enzymatic activity. After activation of the core complex by the protein kinase ataxia telangiectasia and Rad3 related (ATR), the FANCL protein monoubiquinates the FANCD2-FANCI heterodimer (Zhang et al., 2007), which is responsible for translocating into the chromatin foci and removing DNA damaging interstrand crosslinks (ICLs) with the cooperation of other downstream FA proteins (D1, J, N) (Moldovan and D’Andrea, 2009). The most common mutations are found in FANCA, FANCC, and FANCG genes, representing over 80% of all FA cases (Muller and Williams, 2009).

Although it is known that the FA pathway is important for DNA repair, new evidence suggests that this pathway has alternative functions in regard to maintenance of the stem cell pool. Haneline et al. (1999) indicated that FANC-/- mice had a 7 to 12-fold decrease in the ability to repopulate primitive hemopoietic stem cells. Raya and Clevers (2005) demonstrated that the generation of induced pluripotent stem cells from patient samples was only successful when genetic correction of the deficient FANC gene was performed. In addition, in a study evaluating FANCD2 deficient human embryonic stem cells, it was indicated that primitive progenitors formed significantly fewer colonies (Mankad et al., 2006). Based on this evidence, we propose that the loss of the FA pathway may impair HSC properties, specifically the ability to regenerate and repopulate bone marrow. When the FA pathway is compromised, such as in FA patients, the self-renewal
capacity of the HSC’s is altered, resulting in an unfit pool of HSCs that cannot sustain blood production over the lifetime and predisposes the HSCs to leukemia transformation based on selective pressures in the micronenvironment.

Preliminary data suggests that the FA pathway is important in regulating the Wnt (Wingless integration) pathway, a known pathway which plays a major role in the balance of HSC properties such as quiescence vs. proliferation, survival vs. apoptosis, and self-renewal of the stem cell pool vs. differentiation into terminally differentiated cell types. Collaborative work between Dr. Grover Bagby and Dr. Kim-Hien Dao’s laboratories have shown that genes regulating Wnt and Hedgehog pathway activation are aberrantly regulated in FANC-/- KSL cells (in vitro cell line of early hematopoietic stem cells) when compared to FANC+/+ KSL cells exposed to TNF-alpha. This suggests a relationship between the activity of the FA pathway and the regulation of Wnt and Hedgehog pathway activation. It has also been demonstrated that β-catenin, the nuclear effector of the Wnt pathway binds to several of the Fanconi pathway proteins, such as FANCA, C, and G. This suggests a functional relationship between these proteins and the regulation of the Wnt pathway, potentially impacting the fitness of the HSC pool. FANCL is of significant interest because it is an E3 ubiquitin ligase. Although the evidence suggests that the loss of the FA pathway influences the regulation of HSCs, specifically by interactions with the Wnt canonical pathway, it is not clear on how the FA pathway regulates or controls HSC function and the fitness of the HSC pool.

Based on these observations (a tendency to develop hematological cancers such as AML and data suggesting that the Fanconi pathway proteins in some way interact with β-catenin), it is of significant importance to understand whether the FA phenotype results in a
change in the HSCs ability to appropriately self-renew. The specific aims of this study were to validate the ability of shRNA constructs to knockdown FancL protein expression \textit{in vitro} and to evaluate the impact of FANCL knockdown on human cord blood CD34+ colony formation. We hypothesized that the loss of the FANCL protein in HSCs would result in an unfit stem cell pool that would consequently lack the ability to repopulate primitive HSCs. Understanding the consequences of this study on HSC pool fitness is of significant importance because it may provide novel insights into important biological processes concerning cancer transformation.

\textbf{Methods}

\textbf{A. Validation of FANCL shRNA constructs for their ability to knockdown FANCL expression in 293FT cells.}

293 cells, a human embryonic carcinoma cell line, was used to verify knockdown of FANCL by five shRNA constructs numbered 299-302. In a 12-well format, DNA was diluted at a fixed concentration 1ug of DNA to 50ul OM; in a separate tube, lipofectamine2000 was diluted at a fixed concentration of 2ul of L2000 to 50uL OM and incubated for 5 minutes. After this incubation, the diluted DNA and L2000 were combined, mixed gently, and incubated for 20 minutes. After this incubation, media from cells were removed and the DNA/L2000 complexe were added to cells. In general, we used 1.6ug to 2ug of DNA per well.
**qPCR of shRNAs**

293 cells were transfected with either the “Scramble (Scr)” shRNA construct, the negative control (which has a scrambled shRNA sequence that when transfected does not result in knockdown of the gene) or one of the three experimental shRNA constructs. After 48 hours incubation at 37°C, the cells were harvested, pelleted at 365 g for 5 minutes, and then RNA isolation was performed using RNeasy Kit (Qiagen, cat# 74106). cDNA was produced after 24 hours by converting the RNA to first strand cDNA using Superscript VILO Reverse Transcriptase (Invitrogen, cat# 11754-050). qRT-PCR analysis was done with SYBR Green (Invitrogen, cat# 11733-046) as the fluorescent reagent. Each sample was run in triplicate and compared to a GAPDH primer as an expression control.

**Western blot analysis of shRNAs**

Whole cell lysates were generated in NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 1 mM EDTA, 1% protease inhibitor (Sigma Chemical, St. Louis, MO)]. For each condition, 30 µg of protein was loaded, run on SDS-PAGE, and the bands of the gel transferred to a PVDF membrane. Probing was done with anti-FANCL (Santa Cruz Biotech, cat # sc-137067) at a 1:500 dilution for 2 hours at room temperature in 5% milk in TBST. To control whether protein loading was equal for each sample, probing was done with anti-Tubulin (Sigma-Aldrich, cat# T6074) at a dilution of 1:1000. Following this, the blot was probed with anti-Mouse Horse-radish peroxidase conjugated secondary antibody at a dilution of 1:7500 for 1 hour.
B. Transduction of CD34+ cord blood stem cells with FANCL shRNA

Human cord blood was obtained from the OHSU Cord Blood Program that are deemed not suitable for banking purposes and made available to researchres at OHSU. Human cord blood was diluted in PBS and cell clumps were triturated gently. Cells were then pelleted at 1100 RPM for 7 minutes and resuspended in 5ml PBS. The cell number was counted using Guava ViaCount software. Cells were then pelleted and resuspended in 300 µl MACS buffer (0.5% BSA and 500mM EDTA in PBS). For a total cell count of less then $10^8$, 50 µl of blocking reagent and 50 µl of microbeads from the MACS Miltenyi Biotec MACS Separation MS Columns kit for cell selection were added and incubated for 30 minutes at 4°C. Five milliliters of MACS buffer was added, cells were pelleted and then resuspended in 500 µl of MACS buffer. The MACS magnet apparatus was used to select CD34+ cells. Prior to adding the cells, the column was washed with 500 µl of MACS buffer. After the column washing, the whole cell suspension was run through the column. Once the column had completely stopped dripping, the column was washed with an additional 500 µl MACS buffer three times. Once washing was complete, the column was removed from the magnet, 1 ml of MACS buffer was added, and the buffer + CD34+ cells were removed using a syringe into an Eppendorf tube. In a 12-well format, DNA was diluted at a fixed concentration 1 µg of DNA to 50 µl OM; in a separate tube, lipofectamine2000 was diluted at a fixed concentration of 2 µl of L2000 to 50 µL OM and incubated for 5 minutes. After this incubation, the diluted DNA and L2000 were combined, mixed gently, and incubated for 20 minutes. After this
incubation, media from cells were removed and the DNA/L2000 complex was added to cells. In general, we used 1.6 μg to 2 μg of DNA per well.

C. Colony forming assays in methylcellulose

CD34+ cord blood stem cells were transduced with lentiviral particles generated from the Scramble (control) or FANCL shRNA constructs for 48 hours. During this time, cells were grown in Stempro supplemented with TPO (0.1 μg / ml), Flt3L (0.5 μg / ml), IL2 (0.1 μg / ml), penicillin/streptomycin (1 μg / ml), and fungizone (0.25 μg / ml). After 48 hours the cells were washed and pelleted and transferred into methylcellulose (H4434, Stem Cell Technology) with or without puromycin at 1 ug/ml, and containing penicillin/streptomycin (1 μg / ml), and fungizone (0.25 μg / ml). Colony number and type were scored approximately 12-14 days later.

D. Immunofluorescence staining to evaluate beta-catenin and FANCL expression.

293 cells were transduced with either a control gene (LacZ) or with a FANCL gene in order to upregulate gene expression and then cells were exposed to either 1 μM BIO [6-Bromoindirubin-3’-oxime, a selective glycogen synthase kinase-3 [GSK-3] inhibitor which maintains activation of the Wnt signaling pathway in vitro (Sigma-Aldrich, cat# B16861)]; mitomycin C [chemotherapeutic agent that causes DNA damage (Sigma-Aldrich, cat# M4287-5X2MG)]; both BIO and mitomycin C; or neither treatment while in culture. The 293 cells were then plated on poly-I-lysine coated coverslips. After transfection of the shRNA constructs, slides were washed by dipping the coverslips into containers with PBS three times. The cells were then
fixed using 4% paraformaldehyde in PBS for 15 minutes at 37°C. After incubating, the coverslips were rinsed three times using PBS. Permeabilizing the cells was done by adding 0.25% Triton X-100 in PBS and allowing the cells to sit for 10 minutes at room temperature. Coverslips were washed again with PBS. Cells were then blocked with a mixture of 10% goat and donkey serum in PBS for 20 minutes at room temperature, then the coverslips were rinsed with PBS. The primary antibody [β-catenin 1:50 dilution in PBS (Santa Cruz, cat# SC-7199)] was then applied to the cells and incubated overnight at 4°C in the dark. After incubation, the cells were rinsed in PBS, and following the wash, the secondary antibody [FITC CD34 1:11 dilution in PBS (Miltenyi, ca# 130-081-001); Alexaflour555 goat anti-rabbit 1:500 (Invitrogen, cat# A21429)] was applied and the coverslips were incubated for 45 minutes at room temperature in the dark. Coverslips were rinsed in PBS then dipped into ddH\textsubscript{2}O for a final rinse. The coverslips were then mounted onto slides using mounting medium containing 1 µg / mL DAPI and then stored at 4°C.

**Results**

**shRNA constructs have the ability to knockdown exogenous and endogenous expression of FancL.** Western blot analysis of human embryonic kidney cancer cells (293n cell line) transfected with the scramble (Scr) or FANCL shRNAs (299, 301, and 302) show adequate knock down of exogenous expression of FancL *in vitro* when compared to the Scr control (Figure 1a). Exogenous expression of FancL protein was compared to the transfected control (Scr) and to cells transfected with the experimental shRNAs (299, 301, and 302), as well as a mixture of all three shRNAs (299 + 301 + 302). As expected, the
transfected Scr control did not interfere with exogenous expression of FancL, thus was an appropriate control. Knockdown of FancL expression by the shRNAs was observed by the lack of a band indicating adequate suppression of FancL expression within cells. Knockdown of endogenous FancL expression in 293 cells transfected with the experimental shRNAs was quantified and confirmed by qRT-PCR analysis (Figure 1b). The qRT-PCR analysis indicated that the experimental shRNAs decreased endogenous FancL mRNA expression within the cells when compared to the negative control (Scr) by at least 40% by each shRNA construct. Endogenous mRNA (remember this is qRT-PCR not a western blot) expression of FancL was significantly reduced in all three constructs when compared to the Scr shRNA (p < 0.05). These results indicate that all three shRNA constructs can knockdown exogenous expression of FancL as well as endogenous expression of FANCL. The assumption then is that the knockdown of this protein results in the FA phenotype (excessive chromosome breaks or loss of monoubiquitination of FANCD2 with mitomycin C), which we are currently verifying.
Figure 1. (A) Western blot analysis of FancL expression in human 293 embryonic kidney cancer cells transfected with small hairpin RNA (shRNA) constructs. Knockdown efficiency of the experimental constructs (299, 301, 302) was compared to exogenous expression of FancL without any shRNA transfection and to the transfected negative control (Scr). (B) Mean FancL expression by qRT-qPCR in human 293 embryonic kidney cancer cells transfected with shRNA constructs. Cells were cultured until 70-80% confluence and transfected with the appropriate shRNA. After 48 hours of incubation, qRT-PCR analysis was done for cells with each shRNA construct. Experimental shRNA constructs (299, 301, 302) were compared to Scr control for knockdown efficiency. FancL expression with the experimental shRNA constructs was significantly reduced in all three constructs compared to Scr and this is denoted by the asterisk (p < 0.05). Error bars represent standard error for n = 2.

Knockdown of FANCL in cord blood CD34+ cells results loss of multipotent progenitors and a shift towards lineage restricted progenitors. Colony formation assays in methylcellulose indicated that the total number of GM cells (granulocyte and monocyte progenitors) was not reduced when transfected successfully with the shRNA constructs (indicated by selection in puromycin) when compared to the negative control, Scr (Figure 2a). The total number of GEMM stem cells (granulocyte, erythrocyte, monocyte, and megakaryocyte progenitors), a more primitive stem cell, decreased 4-fold in cells transfected successfully with the 301 and 302 shRNA constructs (indicated by selection in puromycin) when compared to cells transfected with the Scr construct. The 299 shRNA construct did
not reduce the ability of those cells transfected with the shRNA to form colonies, however, when compared to cells transfected with Scr (Figure 2b). This observation can be seen when comparing the percentage of GM and GEMM colonies from the total number of colonies counted for all cells transfected with each of the shRNA constructs. The percentage of GEMM colonies formed by unselected CD34+ cells indicated no difference in the ability of colonies to form from cells transfected with all three experimental shRNA constructs (Figure 3a). However, when selected for transfection in puromycin, the percentage of GEMM colonies formed is reduced by an approximate 5-fold decrease in cells transfected with the 301 and 302 shRNA constructs; no change in the colony formation ability for cells transfected with the 299 construct was observed (Figure 3b).
Number of colonies formed from human cord blood CD34+ cells transfected with small hairpin RNA constructs and selected in puromycin. Cells transfected with each shRNA construct were plated into methylcellulose without puromycin and methylcellulose containing puromycin (which selects for transfected cells). Total numbers of colonies formed in the positive puromycin condition were normalized to the negative puromycin condition to take into account transfection efficiency with each treatment. Total number of colonies formed for cells with each experimental shRNA construct was then normalized to Scr to compare to the control. Colonies were identified as either arising from GM (granulocyte and monocyte progenitor) or GEMM (granulocyte, erythrocyte, megakaryocyte, and monocyte) stem cells by visual identification; GM colonies appear clear visually under the scope whereas GEMM colonies appear to have clear and red cells. (A) Total number of GM (granulocyte and monocyte progenitor) colonies in methylcellulose without selection for transfected CD34+ cells and with selection using puromycin for transfected CD34+ cells. There is no statistically significant difference among cells with the experimental shRNA constructs and Scr. Error bars represent standard error for n = 4. (B) Total number of GEMM (granulocyte, erythrocyte, megakaryocyte, and monocyte progenitor) colonies in methylcellulose without selection for transfected CD34+ cells and with selection with puromycin for transfected CD34+ cells. There is no statistical difference in total number of GEMM colonies counted between cells transfected with the Scr control and the 299 shRNA construct. The differences in total number of GEMM colonies counted between Scr and the 301 and 302 shRNA constructs, however, were statistically significant (p < 0.05). Error bars represent standard error for n = 4. Asterisks represent statistical significance.
Figure 3. Percentage of GEMM and GM progenitor cells in total colonies counted from human cord blood CD34+ cells transfected with small hairpin RNA constructs and selected in puromycin. Transfected cells with each shRNA construct were plated into methylcellulose without puromycin and into methylcellulose containing puromycin (selects for transfected cells). The percentage of colonies formed in the positive puromycin condition was normalized to the negative puromycin condition to take into account transfection efficiency within each treatment. Percentage of colonies formed for each experimental shRNA construct was then normalized to Scr to compare to the control. (A) Unselected colony growth as percentage of total colonies counted. Green bars indicate percentage from total of GEMM cell progenitors and red bars indicate GM cell progenitors. Error bars represent standard error. There is no significant difference in the percentage of GEMM cell colonies formed. (B) Selected colony growth in puromycin as percentage of total colonies counted. Green bars indicate percentage from total of GEMM cell progenitors and red bars indicate GM cell progenitors. Error bars represent standard error. There is no significant difference in the percentage of GEMM colonies formed between Scr and the 299 shRNA construct treatments, however, differences between Scr and the 301 and 303 shRNA construct treatments were statistically significant (p < 0.05). Asterisks represent significance.

Increased Wnt pathway stimulation enhances recruitment of FANCL proteins to sites of chromosomal breaks in vitro. On observation, cells overexpressing the control gene (LacZ) and FANCL protein lacked noticeable recruitment of FANCL protein into the nucleus at the sites of chromosomal damage (representative image in Figure 4a). With the addition of mitomycin C exposure, FANCL recruitment was observed in both cells.
overexpressing the LacZ gene as well as FANCL, however, noticeably enhanced recruitment was observed in cells overexpressing FANCL exposed to Wnt pathway stimulation (by BIO treatment) when compared to the control (representative image in Figure 4b). The difference is observed qualitatively and currently, we are working towards quantifying the observations with additional experiments.

Figure 4. Immunofluorescent images of human 293 embryonic kidney carcinoma cells. Cells were transfected with either a control gene (LacZ) or a FANCL then treated with or without BIO (6-Bromoindirubin-3’-oxime, a selective glycogen synthase kinase-3 [GSK-3] inhibitor which activates the Wnt pathway); mitomycin C (chemotherapeutic agent that causes DNA damage); both BIO and mitomycin C; or neither treatment while in culture. Only the control treatment (neither Bio nor mitomycin C) and the experiment with both treatments are shown here. (A) Immunofluorescent image of cells overexpressing FANCL protein without exposure to mitomycin C or BIO. (B) Immunofluorescent image of cells overexpressing FANCL protein with exposure to both mitomycin C and BIO.
Discussion

This work provides a connection between loss of FANCL, a key component of the FA pathway, and decreased multipotent progenitor expansion. This was determined by suppressing FANCL expression using validated FANCL shRNA constructs and performing colony forming assays. It was observed through colony forming cell assays of cord blood stem cells that FANCL-deficient hematopoietic stem cells have a reduced capacity (up to a 5-fold reduction) in forming multi-lineage progenitors. These results are consistent with previous data, suggesting that FANC-deficient stem cells have defective regenerative capacity and impaired repopulation ability. Tulpule et al. (2010) demonstrated that embryonic HSCs deficient in the FA pathway have a remarkably reduced capacity for hematopoietic development, as well as decreased long-term repopulating activity in vitro. Our data which suggests that the loss of the FA pathway may lead to an unfit pool of stem cells which are susceptible to clonal adaption and selection, is also supported by the clinical presentation of the disease, both by the onset of aplastic anemia and leukemogenesis later in life; an unfit HSC pool consequently results in the lack of blood cell production over the lifetime as well as a predisposition for leukemic transformation. According to Mikesch et al. (2007), leukemia stem cells arise from genetic events in HSC progenitor cells where mutations alter transcription and lead to aberrant gene expression, leading to the malignant transformation into a cancer stem cell. The infinite life-span of HSCs makes them an ideal population to acquire mutations required for leukemic transformation, more so when regulatory pathways are disrupted such as the FA pathway (Chao et al., 2008).

For future study, we will investigate whether the Wnt/β-catenin pathway is the mechanistic explanation for why FA-deficient HSCs have impaired pluripotent potential.
The canonical Wnt pathway plays a major role in embryogenesis for the determination of cell types and is also implicated in the maintenance of stem cell properties. According to Fleming et al. (2008), the Wnt signaling ligand is a morphogen that promotes the expansion and maintenance of hematopoietic stem cells by activating transcription factors. Wnt signaling activation is required by HSCs to mediate the quiescent property of stem cells; in very specific and appropriate amounts, Wnt signaling will increase the quiescence and self-renewal properties in order to preserve the steady-state HSC pool and limit differentiation and proliferation into progenitor cells (Fleming et al., 2008). In a study done by Kirstetter et al. (2006), they demonstrated that constitutive Wnt signaling results in no terminally differentiated myeloid cells and an increase in the number of HSCs in transplantation studies done in mice. Furthermore, it has been shown that differentiation of HSCs occurs when Wnt cascade pathways are down-regulated (Scheller et al., 2006). In recent studies done in collaboration with our lab, it has been shown that genes regulating Wnt pathway activation, a known pathway involved in hematopoietic stem cell renewal, are aberrantly regulated in FA-deficient cells. By identifying the relationship between the FA pathway and Wnt/β-catenin signaling, novel insights about HSC renewal properties and maintenance of the stem pool can be determined.

Although promising, these results are limited in their application, since it has not yet been evaluated whether knockdown of FANCL in HSCs results in the FA phenotype, due to limitations in our technique to acquire adequate cell numbers for quantification. The FA phenotype can be determined in cord blood cells by a chromosomal breakage assay, in which the number of breaks in FA deficient cells are quantified and compared to normal cells when treated with DNA damaging agents such as mitomycin C. If the FA phenotype is
manifested in these cord blood cells, then it implies that the shift from a stem cell pool consisting of multi-lineage progenitor cells to a more lineage restricted stem cell pool in FANCL-deficient cord blood cells is representative of what is occurring in FA patients, which can thus be studied in the context of Wnt/β-catenin signaling and the maintenance of the HSC pool.

**Literature Cited**


