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ETS-Domain Transcription Factor Elk1 is Critical for Embryo Implantation via Regulatory Control on Superoxide Dismutase 1 (SOD1)

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Abstract

Mitogen Activated Protein Kinase (MAPK) signaling is one of the decisive pathways in regulating embryo-uterine interactions. Ras-MAP Kinase activation is effected by ETS family proteins; ELK1, being the first member of the Ternary Complex Factor (TCF) family, is rapidly activated by ERK. Based on the significance of ETS family member in the process of embryo implantation and presence of ELK1 during embryogenesis, we hypothesized that ELK1 would also discharge its role in embryo implantation. We intend to analyze the importance of ELK1 during embryo implantation for which we have used a mouse pregnancy model system. Our results clearly document the expression of ELK1 during different days of pregnancy. ELK1 is highly activated during peri-implantation period as its expression in the nucleus is increased compared to pre-implantation stages. *Elk-1* knock down leads to pregnancy failure which is attributed to changes in perforin, *c-fos, Mcl-1* and *Sod1*. *Elk-1* also decreases SOD activity which is the crucial factor controlling superoxide during embryo implantation. These results suggest that *Elk-1* affects the process of embryo attachment to uterus either directly or by way of molecules that are obligatory for the process of embryo implantation.

Keywords: *Elk-1*, Embryo Implantation, SOD1, SOD Activity

1. Introduction

The rate of successful pregnancy is low even after the advancement in assisted reproductive technologies. More than 50–75% pregnancy loss occurs during the time of embryo implantation, which involves a complex series of genetic and cellular interactions[1]. However, the mechanism underlying these processes is still elusive. A better understanding of these complex series of events would help in advanced treatments for infertility and recurrent pregnancy loss. Uterine receptivity leading to successful embryo implantation is restricted to a short period of time termed the "window of implantation"[2]. Understanding the regulation of genes and their expression during the "window of implantation" will be a better approach to identify the regulatory signatures in the process of implantation.

Every step of the embryo implantation is thought to be interceded by a large number of molecules, and many transcription factors modulate gene expression[3]. Transcription factors in the ETS family play vital roles in embryo implantation; they have a conserved DNA-

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binding domain regulating the expression of many cellular and viral genes. Ras-MAPK activation is effected by ETS family proteins in the nucleus regulating the growth-related genes and other immediate early response genes resulting in cell proliferation[4]. They also can regulate gene expression by interacting with other cellular partners. Transcription factors of the ETS family such as PEA3, ERM, ER81, Fli1 and TEL1 play important roles in implantation and subsequent development of embryo[5,6].

Two new members in the Ets oncogene superfamily (*Elk-1* and *Elk-2*) have been reported recently. Multiple roles have been assigned to ELK-1 *viz*. neuronal differentiation, cell proliferation, tumorigenesis, and apoptosis[7]. Presence of ELK-1 during embryogenesis also has been reported by several investigators[8,9]. Based on the background literature highlighting the significance of ETS family member in the process of embryo implantation, we hypothesized that ELK-1 could also discharge its role in embryo implantation. Knock out studies in mice help to understand the mechanism behind human embryo implantation as mice and humans have certain features in common. In this study, we are highlighting the importance of ELK-1 during embryo implantation using mouse as a model system.

2. Methodology

2.1 Materials

Elk1 (I-20) polyclonal antibody (sc-355), and Goat Anti-Rabbit HRP (sc-2030) and *Elk 1* siRNA (m) (sc-35291) were from Santa Cruz Biotechnology, Inc, USA while PVDF (0.45 µm) and BIO-RAD DC Protein Assay kit were from Bio-Rad Laboratories Inc. Goat Anti-Rabbit Alexa Fluor 488 (A11070), Propidium Iodide and other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Animal Models

2.2.1 Pregnant Mice Model

Experiments were performed on regularly cycling sexually mature 3-5 month old virgin female mice, and tissues were isolated on different days of "window of implantation" as detailed elsewhere[10]. In addition, inter-implantation and implantation sites of the peri-implantation uteri were separated after intravenous injection of 1% Evans Blue dye *via* tail vein, 15 minutes prior to the sacrifice of animals, and stored separately at -80°C until further experiments.

2.2.2 In vivo Silencing Model

For *in vivo* gene silencing, the female mice received an intra-luminal injection of *Elk1* siRNA (sc-35291, Santa Cruz Biotechnologies, Inc., USA) at the dosage of 1.96 nM per day/25 g wt of mouse on day 3, 4 pm and the animals were sacrificed on Day 5, 5 am as detailed[11]. The uterine horn was ligated at one end to avoid transfer of the contents between horns; the control siRNA (sc-37007, Santa Cruz Biotechnologies, Inc., USA) of the same dosage was injected into the contralateral horn.

Another set of animals in this model was used for intra-peritoneal injection of *Elk*-1 siRNA. Two dosages were administered till the thirteenth day of pregnancy, and animals were sacrificed on day 14. The animals were sacrificed either on Day 5, 5 am or on Day 13, 10 am. The Day 5,5 am mice uteri were processed for RNA or protein extraction after flushing out the embryos.

2.3 RNA Extraction and Real Time Analysis of *Elk1* Expression

Total RNA was extracted from mice uteri of different days of pregnancy using Trizol reagent (Sigma, St. Louis, MO, USA). The RNA was converted into cDNA using SuperScript™ III First strand synthesis system for RT-PCR (Invitrogen, USA). The samples were normalized using GAPDH as the house keeping gene. qRT analysis was done on 7900 HT Fast Real Time PCR System (Applied Biosystems, USA) with 25 ng of cDNA using POWER SYBR green PCR master mix (Applied Biosystems) and $2^{-\Delta\Delta CT}$ method was used for relative quantification of the genes. All samples were done in duplicates and the experiments were repeated thrice. Primers for real-time PCR were made from Eurofins Genomic India Pvt. Ltd. (Bangalore, India). *Elk1R: 5'ATGGCCGAGGTTACAGACAC3'; c-FosF:AGT CAAGGCCTGGTCTGTGT, c-FosR: TCCAGCACCAGGT TAATTCC, PerF:GAATGCAAGCAGAAGCACAA, PerR: TGTGTGTTCACTGGGAAGGA; Sod1F:GAGACCTGGG CAATGTGACT, Sod1R:TTGTTTCTCATGGACCACCA; Mcl1F:TAGAAGGCGGCATCAGAAAT*

2.4 Cytosolic and Nuclear Extract Preparation

Cytosolic, nuclear and membrane proteins from the mice uteri were extracted using CelLyticNuCLEAR Extraction Kit (Sigma) as detailed by manufacturer. In short, the tissues were minced and homogenized at 15,000 rpm (3 strokes/30 seconds) using a polytron homogenizer at 4°C in 1X Lysis buffer. The homogenates were incubated for 60 minutes on ice on Genei Rocker-100 and centrifuged in Eppendorf 5820R centrifuge at 1000 X g at 4°C for 45 minutes to pellet the nuclei. The supernatants obtained were stored at -80°C as cytosolic extracts. Nuclear proteins were extracted from the nuclear pellet by adding extraction buffer, and after vortexing they were incubated on ice for 30 minutes on rocker followed by centrifugation for 30 minutes at 20,000 X g. The supernatants were stored as nuclear extracts at -80°C.

2.5 SDS-PAGE and Western Blotting

Thirty micrograms protein from the cytosolic or nuclear extracts of different stages of pregnancy were diluted 1:2 with Laemmli sample buffer and blotted onto PVDF membrane after resolving on SDS-PAGE gels as detailed elsewhere[10]. Blots were developed either as per earlier protocol[10] with 5% milk as blocker and appropriate primary and secondary antibody or signals were measured by enhanced chemiluminescence detection (Bio-Rad, USA) and visualized by X-ray radiography.

2.6 Immunocytochemistry on the Pregnant Uterine Sections

The excised uteri were processed for sectioning and immunolabeling as described earlier[10]. Minor modifications included use of 1% BSA as blocker, overnight incubation with primary antibody (1:100) at 4°C and incubation with secondary antibody (FITC labeled) (1:200) for 60 minutes and Propidium Iodide was used to stain the nuclei. Images of the uterine sections were captured on Leica SP2 confocal laser scanning microscope.

2.7 SOD Activity Assay in *Elk*1 Silenced Cells

To confirm if the *Elk1* silencing affects SOD activity, we performed the activity assay in *Elk1* silenced and control silenced cells using SOD assay kit from Sigma as per manufacturer's instructions.

3. Results

3.1 Expression Profiling of *Elk-1* "During Window" of Implantation

Expression profiling of *Elk-1* by relative quantification using real-time PCR shows a differential expression of *Elk-1* during the "window of implantation" (Figure 1A). Highest level of expression of *Elk-1* was observed at peri-implantation Day 5, 5 am stage as compared to the early peri- implantation stage, i.e., Day 4, 4 pm and pre-implantation stages Day 4, 10 am. A fall in the expression of *Elk-1* was seen in the post-implantation stage, Day 5, 10 am. The results show that the expression of the *Elk-1* gradually increases as the stage of pregnancy advances till the peri-implantation stage and decreases after the process of embryo implantation. The change in expression was statistically significant in all the groups compared viz., $p<0.03$ between Day 4, 10 am and Day 4; 4 pm, $p<0.00001$ between Day 4, 4 pm and Day 5, 5 am; and $p<0.0003$ Day 5, 5 am and Day 5, 10 am.

3.2 Immunohistochemical Localization of the ELK-1 Protein in the Pregnant Uterine Tissue Sections

Immunohistochemical observations of the pregnant uterine sections of different days of pregnancy gave us better understanding into the spatio-temporal distribution of the protein in the uterus during pregnancy. We visualized a cytosolic expression of the protein in the pre-implantation stage i.e., at the Day 4, 10 am and the trend continued in the early peri-implantation stage, i.e., the Day 4, 4 pm stage (Figure 1B). A noteworthy up-regulation of the protein was visualized in the implantation sites of the peri-implantation stage i.e. the Day 5, 5 am (Day 5, 5 am IMP) (Figure 1B). The most striking observation was the pronounced nuclear presence of the ELK1 protein in the implantation sites of the peri-implantation uterus (perfect yellow color of merge areas of nuclear (PI) staining and ELK1 FITC fluorescence) when compared to the inter-implantation zones (orange color) which revealed lowered expression of the protein in the inter-implantation site of the peri-implantation stage (Day 5, 5 am IIMP) (Figure 1B). The

protein continued to be retained both in the cytosol and the nucleus during the post-implantation period also, i.e., at Day 5 (10 am) with export from nucleus visible due to orange color in the merge image. These observations indicate that ELK-1 is expressed in significant amounts in the nucleus of pregnant uterus at the peri-implantation stages.

3.3 Nucleo-cytosolic ELK-1 Expression Levels in Pregnant Uterine Extracts

In order to analyze the ELK-1 expression, uterine cytosolic and nuclear extracts were prepared as per methods and analyzed using SDS-PAGE and western blotting**.** Nuclear extracts from different days of pregnancy reveal a very significant expression of the protein in the later stages of the pregnancy i.e., towards the Day 5, 5 am. A marginal increase in expression is seen at Day 4, 4 pm ($p<0.05$) when compared to Day 4, 10 am. However, as the "window of implantation" progress to peri-implantation stage, significant up-regulation of protein was observed in the nuclear extracts ($p<0.05$) and a significant down-regulation was observed at post-implantation stage, Day 5, 10 am ($p<0.05$). Histone H3 was used as the loading control for nuclear extracts (Figures 1C and E). In the cytosolic extracts, an accrual in the levels of ELK-1 expression is seen in Day 4, 4 pm ($p<0.05$) which increases to higher levels at peri-implantation (Day 5, 5 am) stage as compared to the early peri-implantation stage i.e. Day 4, 4 pm ($p<0.05$) and pre-implantation stages Day 4, 10 am. A fall in the expression of the protein was seen in the post-implantation stage, Day 5, 10 am. However, the variation was not statistically significant. Beta actin

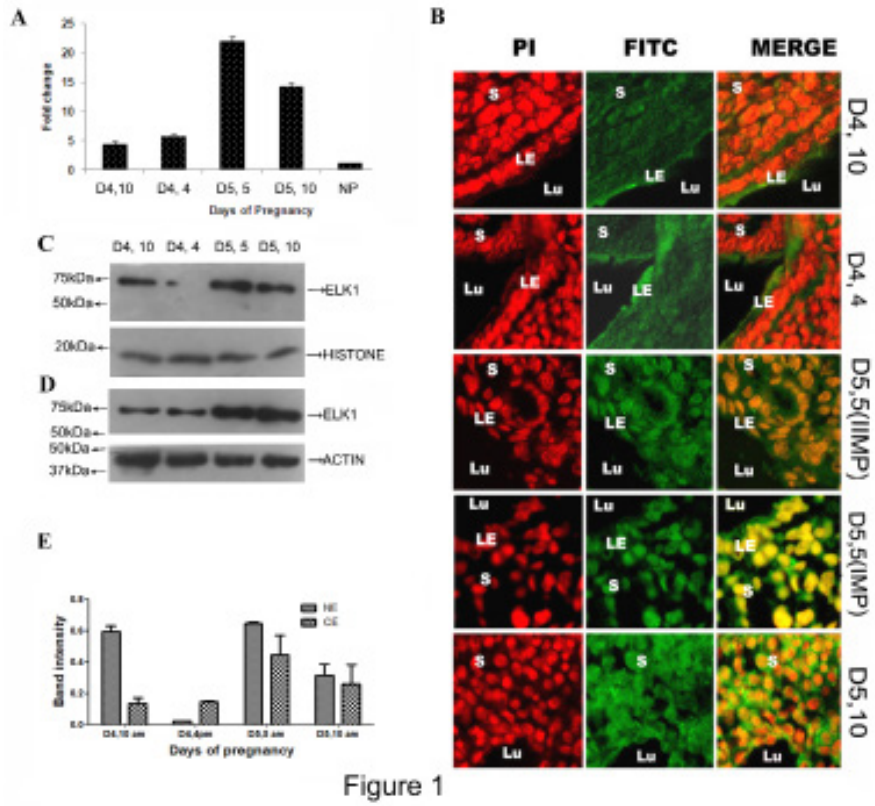

Figure 1

Figure 1. Elk1 is expressed in the uterine nucleus during the window of embryo implantation. **A.** Relative fold change in *Elk1* expression during different days of pregnancy. **B.** Immunofluorescence analysis of ELK1 (green) with nuclear staining (DNA, red) in mouse uterus during pre-implantation stage (D 4, 10 am), early peri-implantation uterus (D 4, 4 pm), inter-implantation site of late peri-implantation uterus (D 5, 5 am IIMP), implantation site of late peri-implantation uterus (D 5, 5 am IMP) and post-implantation uterus (D 5, 10 am). **C–D**. Western blot analysis of ELK1 expression in mouse uterine nucleus (C) and cytosol (D) shows upregulation and distinct nuclear expression at Day 5, 5 am in mouse uterus. Actin and Histone H3 are the loading controls. **E.** Histogram showing ELK1 protein levels in nuclear and cytosolic fraction.

was used for normalizing the expression levels (Figures 1D and E). These results show that the expression of the protein gradually increases as the stages of pregnancy advances. This is coinciding with the real-time PCR data and immunohistochemical data which show higher expression of ELK-1 at peri-implantation stage (D 5, 5 am).

3.4 Effect on Embryo Implantation by the Use of *Elk-1* Si RNA

To understand the influence of ELK-1 protein in the process of embryo implantation, we used the siRNA against *Elk-1* to see the effect of the same in embryo implantation. From the results obtained, we see an absolute blockage of implantation in both the cases when the intraperitoneal and the intraluminal injections were given. The control animals showed implantation (Figure 2A). Expression analysis of *Elk-1* in knockdown uteri shows that more the 86% percentage knock down was observed after *Elk-1* siRNA administration, when compared to D 5, 5 controls (Figure 2B). In order to confirm the silencing of the *Elk-1,* western blot analysis using nuclear and cytoplasmic extracts was performed. Data clearly indicate the absence of ELK-1 in both the cytoplasmic and nuclear compartments (Figure 2C). Nuclear extract showed two predominant immunopositive bands of ~75 kDa and ~42 kDa as shown in Figure 2C. As per the previous reports, as ELK-1 of ~42 kDa was present in rat brain[12]. However, in cytoplasmic extract, a prominent band at ~42 kDa position was not detected.

3.5 Identification of the Molecules that are Regulated by ELK1

To have a better insight of the mechanism of action of ELK-1, we studied the expression pattern of the molecules that are regulated by ELK-1. Microarray studies revealed that the genes that have the promoter binding site for ELK-1 are mostly in the T-cell antigen receptor pathway. The ELK-1 binding sites were present in the promoters of 13 genes in the pathway[13]. Besides, early response genes such as c-Fos and Mcl-1 are under the regulation of ELK-1[14,15]. We tried to look at the expression of some downstream molecules that are directly or indirectly regulated by the ELK-1 or the ETS family members in the siRNA-treated animals which are cited to be involved in the process of embryo implantation. *cfos, Mcl1, Perforin* and *Sod1* showed down regulation (Figures 3A and B) in Elk1 siRNA treated samples.

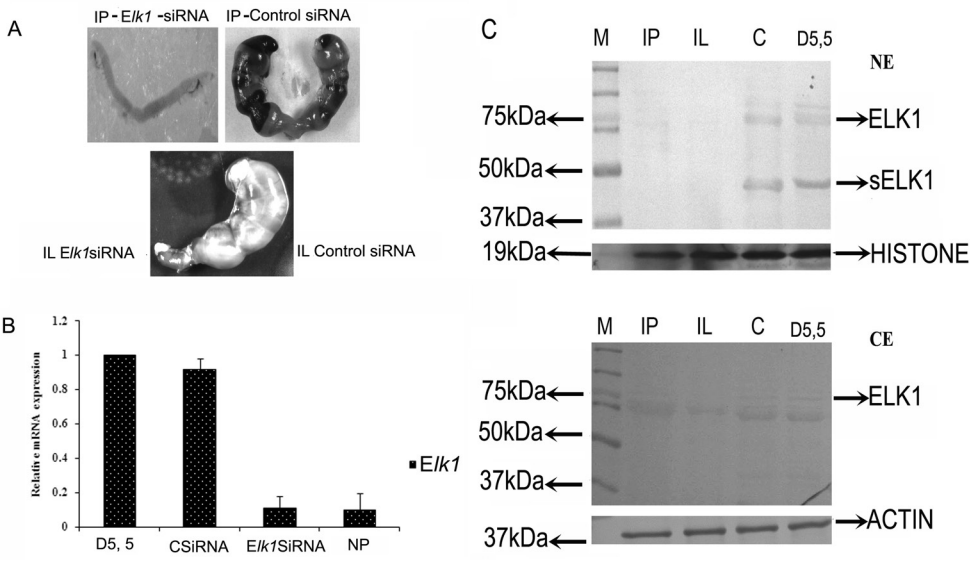

Figure 2. Knock down of *Elk1* in pregnant mice uterus abrogates pregnancy. **A.** Day 13 uteri showing impaired embryo implantation on silencing *Elk1*. siRNA was given intraperitoneally and intra-luminally. **B.** Real time analysis showing *Elk1* down regulation in siRNA treated samples. **C.** Western blot showing ELK1 expression in nuclear and cytosolic extracts of antisense injected uteri. Loading controls, actin and histone H3 are shown below corresponding fractions. M-marker, IP-intraperitoneal, IL-intra-luminal, C-control.

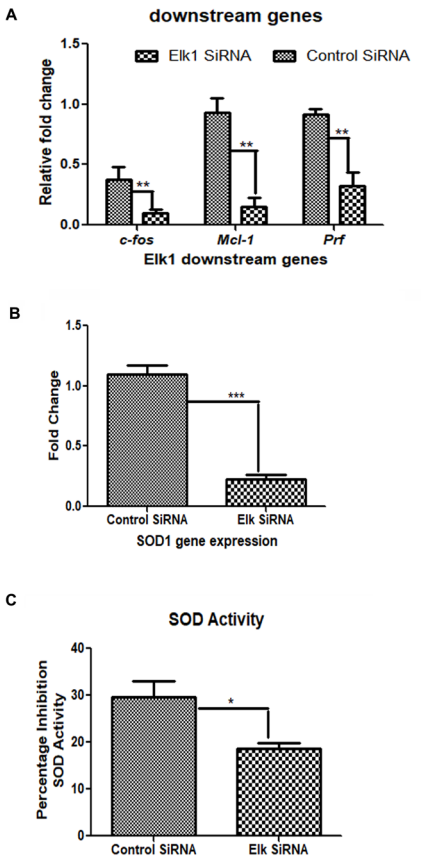

Figure 3. Silencing of *Elk1* affected *Elk1* downstream genes: **A.** Real time analysis of downstream genes of *Elk1* in *Elk1*-silenced mouse uterine samples- *c-fos, Mcl1* and *Perforin*. **B.** *Sod1* gene expression. **C.** SOD activity in *Elk1* silenced RL 95-2 cells.

3.6 SOD1 Activity is Regulated by *Elk-1*

To confirm the importance of *Elk1* in regulating SOD activity, SOD activity assay was performed in *Elk1* silenced MCF-7 cells. Compared to the control siRNA treated samples, the *Elk1* siRNA treated samples showed clear down regulation of SOD activity ($p<0.0410$) suggesting that the earlier reports of promoter regulation of SOD1 by Elk1 also impact SOD activity[16].

4. Discussion

Embryo attachment and implantation are accompanied by several cellular and functional changes in the endometrium. Several ETS family members have been reported to regulate the gene expression during growth and development[4]. ELK1 is the primary member of the TCF family; it acts together in a complex with the transcription factor Serum Response Factor[17]. ELK-1 was initially classified as a proto-oncogene, and subsequently came to be known to control varied biological processes including cell cycle progression and differentiation, tumorigenesis, apoptosis and neuronal differentiation. Elk 1 can act as transcriptional activator as well as repressor at different genomic locations in human embryonic stem cells[18]. Maekava *et al.* show that ERK1 and ELK1 are phosphorylated during mouse peri-implantation stage embryos[19]. Also, report by Banerjee at al. shows that chorionic gonadotropin activated PI3K-Erk-Elk1 pathway helps in preparing uterine endometrium for embryo implantation by regulating prostaglandin synthesis by endometrial epithelial cells[20]. However, so far no report about the role of ELK-1 during embryo implantation is available. Our experiments demonstrate the presence of ELK-1 in mouse uterus during embryo implantation.

Our immunohistochemical data clearly shows the presence of ELK-1 in the implantation site of the uterus

which indicates the importance of ELK-1 during embryo implantation. Real- time PCR data and western blot data in the pregnant uterine extracts very clearly reveal increased expression of ELK-1 at the peri-implantation (Day 5, 5 am) stage. It appears that low amounts of ELK-1 are present in the cytosol or nucleus of the preimplantation (Day 4, 10 am) and early-peri-implantation (Day 4, 4 pm) stage. Increased expression of ELK-1 during peri-implantation stage indicates that ELK-1 plays a crucial role in embryo implantation. Since there is an upregulation of ELK-1 during the time of embryo implantation, we want to know if ELK-1 is an essential gene in implantation. Knockdown studies using *Elk1* siRNA show implantation failure in both mice when we injected the siRNA intra-peritoneally and intra-uterine. Real time PCR shows more than 89% knock down in the expression of *Elk1* mRNA and complete absence of proteins in nuclear and cytoplasmic compartments. However, the administration of control siRNA didn't change the expression and there was no implantation failure also. These indicate that ELK-1 is an essential gene during implantation process.

We have analyzed some of the genes the promoters of which contain*ELK-1* binding site[13]. ELK-1 has also been show to play a role in regulations of SOD1 expression by binding to *Sod1* promoter region[21]. Besides, early response genes such as *c-Fos* and *Mcl-1* are under the regulation of ELK-1[14]. In order to analyze the expression of genes which are regulated by ELK-1, expression profiling of selected genes were performed by real- time PCR using *Elk1* knockdown mouse uterine RNA. We could see the down regulation of *c-fos, Mcl1, Sod1* and *Perforin* expression after *Elk1* knockdown during implantation.

ELK-1 coordinates varied extracellular signals and induces the immediate early gene expression. In response to GH stimulation, the ELK-1 and SRF bind to the Serum Response Element (SRE) for transcription of c-fos proto-oncogene. ERK phosphorylates ELK-1 and Sap-1/2 to promote c*Fos* transcription[22]. Estrogen and progesterone regulate the expression of FOS in the mouse uterus and helps in uterine epithelial cell proliferation. c-Fos expression was also observed in the late pre-implantation mouse embryo encapsulated in zona pellucida.

Mcl1 is an anti-apoptotic member of BCL1 family. EGF-mediated up-regulation of Mcl1 was inhibited during ELK-1 knock down[23]. Mcl-1 deficient embryos fail to implant *in utero*[24].

Perforin is a cytotoxic molecule expressed by Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells to lyse infected or malignant host cells. Perforin was found to be expressed high at feto-maternal interface. No perforin was present before embryo implantation; however, large number of GMG cells was observed after implantation which coincides with decidualization of uterus. This protein has been reported to be expressed in the different endometrial phases aiding in stromal cell breakdown during menstruation and hence important

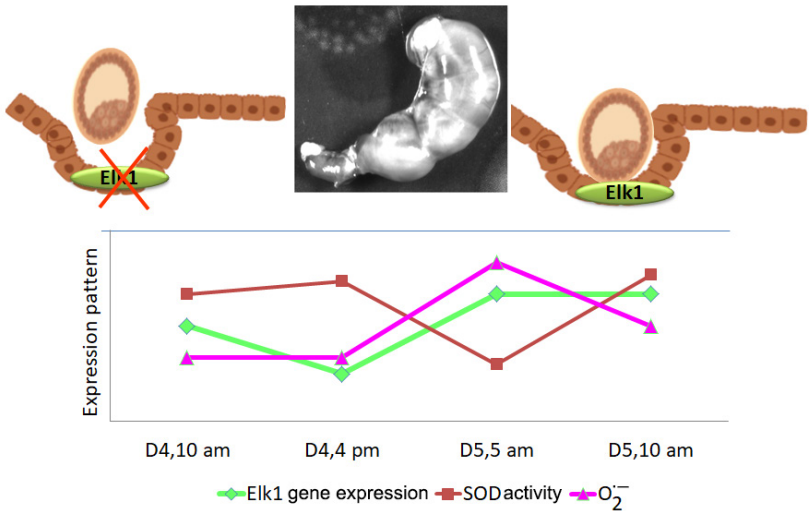

Figure 4. Model depicting mechanism of *Elk1* action during embryo implantation: Absence of *Elk1* in endometrium abrogated pregnancy. The graph represents the expression pattern of SOD activity and Superoxide radical during the "window of implantation" reported by Laloraya *et al.*[28]. As an addition, the SOD activity is controlled by *Elk1* since reduced *Elk1* at early peri-implantation is marked by an increase in Superoxide during peri-implantation period thereby aiding embryo implantation.

in maintaining the function[25]. It has also been reported that perforin plays an immunomodulatory role by down regulating the T cells[26]. CTL-specific expression of perforin gene was regulated transcriptionally by Sp1, an ETS-related transcription factor[27]. Our reports imply that ELK-1 modulates the expression of perforin during implantation.

SOD1 is regulated transcriptionally by Elk1. ELK1 directly binds to the PRE of the *SOD1* promoter, and also forms ternary complex at SRE with SRF to activate transcription of several genes, including *cfos*[16]. It was reported earlier by Laloraya *et al.* that a sharp rise of superoxide radical and a decline in SOD activity was observed during embryo implantation, followed by a rise in SOD activity with a corresponding post-implantation reduction in superoxide radical[28]. We propose that the increase in expression of Elk in the nuclear fraction during peri-implantation is to regulate this sudden rise and fall of superoxide radical and SOD during embryo implantation as loss of Elk1 leads to defective implantation (Figure 4).

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7. Author Contribution

Project conception, design and supervision: ML. ST contributed to Figure 1B, E and 2A. NL contributed to Figures 1A, 2B, 3A-B. ST and NL contributed to Figure 2C. APR contributed to Figures 1C–D, 3C, 4. ST, NL APR wrote the initial draft paper and ML critically reviewed and edited the MS. All authors discussed the results and commented on the manuscript.

8. Conflict of Interest

The authors declare no competing financial interests.

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