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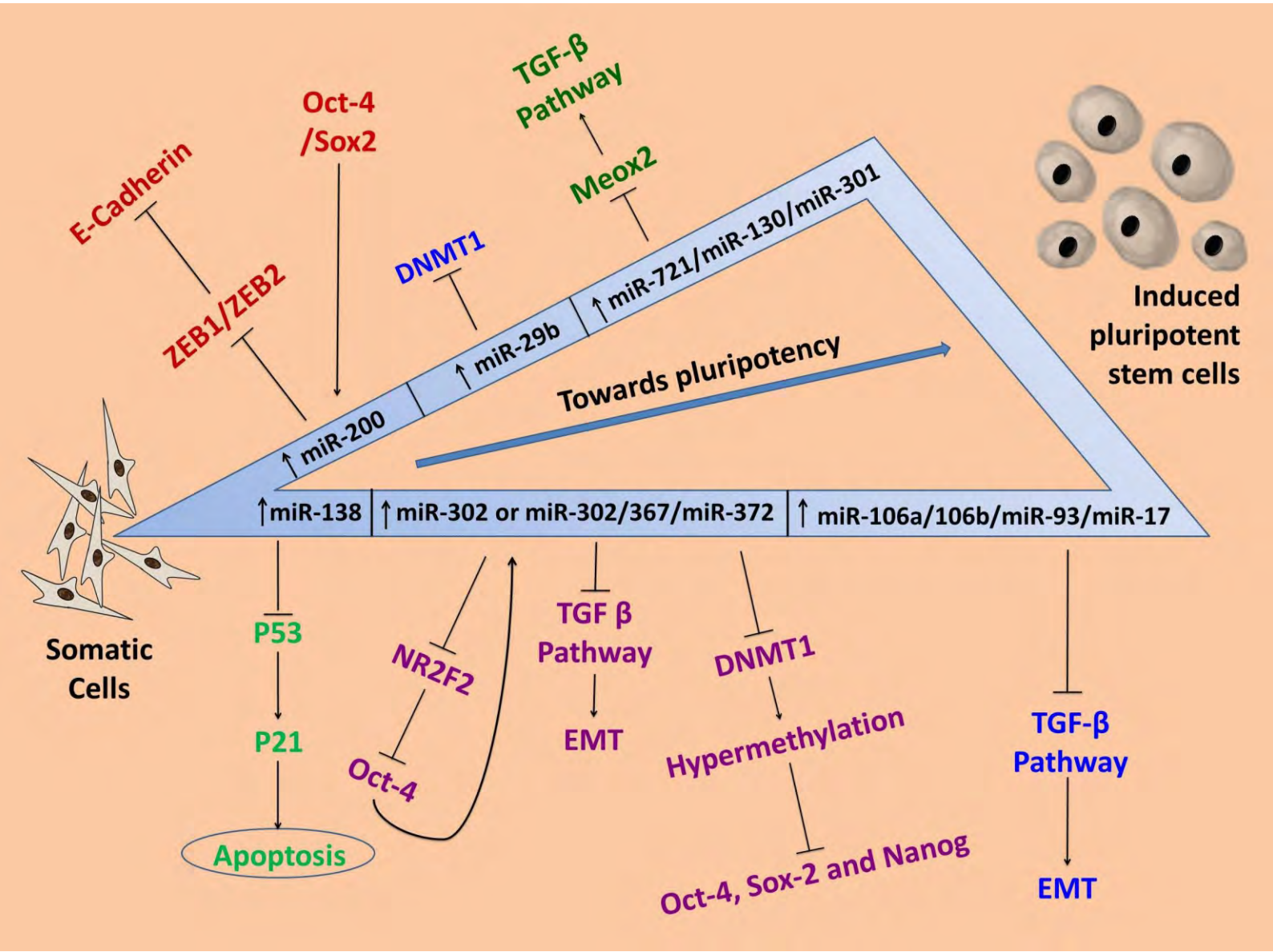

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General Information

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"Biomedical Research Journal (BRJ)" is a premier peer reviewed open access journal, published by NMIMS School of Science for promoting the advancement of ideas in the interdisciplinary realms of Medicine, Science and Technology. The goal is to share new discoveries and translational knowledge with scientists, academicians, clinicians and students in the field of Biomedical and Biological/Chemical/Biotechnology/Stem Cell Biology/Cancer Biology in the realm of basic and applied aspects in the different areas.

BRJ aims at creating a platform to help advance the domains and frontiers of inter- and multi-disciplinary research across the various areas of sciences and recent advances in cross pollination across biology, chemistry, and medicine. Integrative science is the present and future of science, and the various aspects of the journal proposes to highlight and emphasize contemporary technology towards understanding various aspects of the sciences.

The initial focus areas of BRJ include review articles and original research papers in cancer biology, stem cell biology, nano-technology and phytochemistry.

A rigorous peer review process is implemented to judge the effectiveness, legitimacy and reliability of the research content. The papers will be published online as well as provide hard copy of the Journal issues to the authors of the papers on request.

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Editorial

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Advances in Biotechnology and Implications in Clinical Medicine

Dhananjaya Saranath and Aparna Khanna

We continue with the theme of contemporary topics in this issue with articles in stem cell research which has undoubtedly evolved as the most fascinating area of science in the last decade. The current issue initiates an invited editorial on technology with Dr. Tania Fernandez, earlier a Director on the Board of Burill and Company, USA, and currently the Founder and CEO of DreamCatcher Ventures, USA, with her expertise in assessment of biotechnology advances and applications for venture funding, bridging the gap of current state of art technology, scientific basic research and clinical applications, and highlighting translational research from 'Bench to Bedside'. The importance of technology in today's biomedical research is apparent. The 2014 Nobel Prize winners, Eric Betzig, William Moerner and Stefan Hall, were awarded the prize for use of fluorescence to magnify microscopic images to visualize molecules inside living cells. The applications of the technology developed by the nobel laureates enabled a paradigm shift in microscopy to nanoscopy with immense applications including detailed working of cells such as synapses between brain cells, macromolecular aberrations in diseases at cellular level, tracking functioning of cells. The super resolution microscopy/nanoscopy has revolutionised imaging. The current Next Generation Sequencing (NGS) with its myriad applications in disease transmission, epidemiology and clinical applications available and affordable for Predictive, Diagnostic, Prognostic, the right drug and right dosage for individual patients, taking us rapidly into personalized medicine, may be another such technology. We do see the flip side today including finding the needle in a haystack with the NGS mega data analysis; ethical dilemmas of 'To tell or not to tell' with inherent socio-psychological issues; counseling the patients and care givers. A close connect between clinicians requesting tests and answers, and the lab Director providing the tests, for appropriate interpretations has to be mandatory; and the correct pricing in the market for affordability by a patient. Dr. Fernandez takes us through these facets in her editorial. The current opinion is NGS will practically take us into expanded genetic testing in the real world and soon.

We have excellent review articles, and a

method to refrain from use of animal fetal bovine serum for maintenance and proliferation of stem cells for clinical use. Stem cells in regenerative medicine have been a perennial topic for intense discussion and debate around the world. The enormous potential of stem cells in replacement of damaged and diseased tissues in several areas including cardiovascular diseases, neurological diseases, spinal injuries, geriartric diseases like Parkinsons and Alzheimers, genetic diseases, and cancer, makes it feasible to finding cures and treatments for several as yet 'Uncurable' diseases. The regenerative stem cell treatment in the near future is a hope for better quality of prolonged life for the suffering millions. Recently, with the advent of the somatic cell reprogramming technology, the path-breaking noble prize winning work of John Gurdon and Shinya Yamanaka has opened avenues for customized stem cell based therapies. The methodology has obviated the need to use supernumerary embryos, an ethical issue in human embryonic stem cell research.

Despite the tremendous progress in stem cell research and therapy, certain challenges need to be overcome for realizing the ultimate potential of the highly specialized and unique stem cells. This includes improper differentiation to relevant cell types, need for suitable biocompatible matrices to support cell growth, guided delivery of the cells, and mechanisms to comprehend stem cell fate decisions in biological systems. A critical aspect of regenerative medicine should include the stem cell niche and its implications in harnessing endogenous stem cells for successful therapeutic purposes. To address these challenges, interdisciplinary bioengineering strategies for stem cell differentiation; synthesis of novel biocompatible biomaterials for clinical use; sensitive biomedical imaging techniques, are a dire need of the hour.

The review presented in the current issue of the journal by Kanitkar and Kale, discusses the progress in the field of stem cells and tissue engineering. The authors discuss the importance of maintenance of a suitable environment that mimics the *in vivo* natural milieu, for proper differentiation and preservation of the architecture of the cells. Thus, taking cues from the natural environment, generation of mature cell types would depend on a combination of cells, growth factors and a suitable three dimensional (3D) environment to maintain functionality. Scaffolds are biomaterials that are seeded with cells and provide a provisional 3D physical template upon which the seeded cells build new 'Extracellular Matrix' (ECM) and form the regenerated tissue. The ECM of mammalian tissues performs a variety of functions including anchoring of cells, providing a structural support and is supercritical for tissue development, homeostasis and repair. Thus, a key goal to

success in tissue engineering is to effectively emulate several aspects of normal tissue development and remodeling. The article provides a comprehensive review of the various ECMs/scaffolds used for differentiation and growth of stem cells for treatment of a number of medical conditions. Every tissue and organ including bone marrow, have a defined microenvironment or niche that regulates quiescence, proliferation and/or differentiation of the cells within. The authors speculate on the role of 'cellular secretome' for therapeutic purposes, during construction of an artificial niche in their review.

The article by Raut and Khanna reviews the progress in understanding the role of microRNAs (miRNAs), an innate regulatory group of molecules in somatic cell reprogramming. The article briefly describes various approaches used for derivation of induced pluripotent stem cells (iPSCs), and the limitations in existing methods viz., low efficiency of reprogramming, use of harmful oncogenes, and viral vectors for reprogramming. The authors elaborate distinct sets of miRNAs expressed in the early stage and late stage of reprogramming, which play key functions in the reprogamming event. Further the authors highlight the role of miRNAs *per se* in reprogramming, which may culminate in a safe, effective method for efficient reprogramming, moving closer to clinical translation.

An important consideration for translating stem cell therapy to clinics is to avoid use of animal proteins and other macromolecules in processing the cells. The article by Limaye and co-workers, indicates alternative, inexpensive use of cord blood plasma (CBP) as a substitute to the often used fetal bovine serum (FBS) for cultivation of mesenchymal stem cells (MSCs) derived from cord tissue. Normally, cell culture procedures utilize FBS as cell culture supplement to isolate and expand MSCs. However, the individual variability in each batch, and the undefined factors coupled with xenogeneic conditions, necessitates use of 'humanized' cell culture protocols, to facilitate clinical translation of MSCs. An acceptable substitute is cord blood derived AB positive plasma (CBP). The authors describe the *ex vivo* expansion of cord derived MSCs with CBP. MSCs derived from cord tissue represent a promising source for clinical applications. A critical concern in applications of umblical cord blood derived MSC is development of a consistent and reproducible method for in vitro expansion of the cells. The authors demonstrate that MSCs cultured using CBP retain their phenotype, characteristics and differentiation potential. Thus, the authors convincingly demonstrate CBP as a promising alternative to FBS and provide a GMP-compliant protocol.

The stem cell series of articles culminate with Dr. Pandey and his colleagues synopsizing the implications of cancer stem

cells in radiotherapy in cancer, highlighting the tangible reality of clinical applications of stem cell therapy. The review focuses on the critical role of Cancer Stem Cells (CSCs) during radiotherapy. Since irrefutable proof of existence of CSCs has been demonstrated in several cancers, research is directed to this population of stem cells, in an effort to better understand the mechanism of recurrence of tumors after surgery and chemo/radio-resistance. The review also highlights prospects for targeting the CSCs for sensitization during cancer radiotherapy for better prognosis of the disease.

An often neglected area in medicine globally, and in India with a large population of 1.2 billion, is 'Prevention'. With exception of common pediatric vaccines for polio, diphtheria, pertussis, small pox, measles and mumps, disease prevention is not a common practice in India. Further, 'Preventive Medicine in Cancer' is also rarely practiced, despite the consistent increase in the incidence and prevalence of the various cancers. Screening through 'Primary Prevention' including activities that prevent diseases from occurring, and 'Secondary Prevention' that includes screening for diseases at early stages to reduce mortality and improve the quality of life, is a fairly difficult proposition as a systematic, planned program in countries with a large population. Despite availability of tests for screening the normal population and predicting risk for cancers, availability of screening tests and vaccines unequivocally associated with cancers – cervical and hepatic cancers, use of the preventive measures is not a well accepted and also not a feasible proposition, regret to add in India as well. Dr. Maru gives a comprehensive review on 'Cancer Prevention Approaches'. He succinctly puts forth facts such as a majority of cancers are caused, mediated and modified by environmental and lifestyle factors. Changes in lifestyle habits and socially accepted customs, reducing use of tobacco, alcohol, overweight and obesity, have met with limited success and perhaps limited efforts, despite great potential. On the other hand, the numbers of chemopreventive agents and approaches have been limited. The review is a must read for all. The take home message is a healthy lifestyle, use of vaccines where available and appropriate, and regular clinical check-ups to prevent almost 50% of all adult cancers.

Guest Editorial

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Next Generation Sequencing in Healthcare

Tania Fernandez

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The year 2013 was an eventful year witnessing revolutionary discoveries in the world of extraordinary medical advances and healthcare technology. We saw a spate of new and promising discoveries ranging from detecting lung cancer with a cough, pancreatic cancer with accurate faster, cheaper paper diagnostics, to the possibility of using the Human Immunodeficiency Virus to treat genetic disorders in children (Radcliffe, 2013). Emerging newer technologies have fuelled the momentum of the 'genomic revolution'. The completion of sequencing of the human genome project in 2003 (National Human Genome Research Institute, 2010), translated into the rise of the 'omics' era creating mega scientific data. It also gave rise to a breed of genomic companies that focused on application of the emerging technologies, particularly in medical science.

Deciphering the Genetic Code: DNA Sequencing Update

The ability to sequence DNA represented a breakthrough milestone in DNA research. The Sanger sequencing method, developed by the Nobel laureate Frederick Sanger, became the most widely used DNA sequencing method. Sequencing is a way of 'reading' DNA molecules, two complementary strands coiled together to form the double helix. The entire human genome contains about 3.1 billion molecular base pairs per set of chromosomes in a cell.

The story of genome sequencing was not a single 'eureka' moment characterizing the Archimedes discovery, but a compelling story of unbridled passion and continuous advancements pushing the frontiers of genomic technology into interdisciplinary amalgamation of science and technology including advanced materials, nanotechnology, biology, chemistry, enzymology, modeling, and mega data understanding. The story of genome sequencing is one of war and price. The Human Genome Project was one of the costliest 'contests' ever held, a multibillion-dollar government-led effort in the Reagan era, to sequence the entire human genome

Key words: DNA sequencing, human genome project, genomics research.
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which was nearly beaten to the punch by a private company called Celera. The race to sequence the genome became the subject of The Genome War (Barbujani, 2004).

In 2001, the cost to sequence an entire human genome was USD 100 million (National Human Genome Research Institute, 2014). Since then, the cost has moved swiftly downwards and indicated USD 1 million around 2007 when the genome of Nobel laureate Professor James Watson was sequenced. The price has continued on its downward curve, falling to about USD 3,000–5,000 in 2013, although specialized sequencing for cancer patients often costs more. While researchers, companies and investors still argue and agonize on costs of sequencing the human genome, there is no disputing that the pricing has rapidly plummeted downwards.

Genome analysis is one of the fastest-emerging fields in the world, with the recent pricing close to passing USD 1,000, a milestone (Herper, 2014), and continuing to decelerate. The race is on to reach a price of USD 100 per complete genome. Cutting-edge 'next-generation sequencing', allows a greater throughput by parallelizing the sequencing process and producing millions of sequences concurrently. So-called third generation sequencing methods have since supplemented the second generation sequencing methods enabling a greater throughput while at the same time reducing the time to result and costs. Third generation sequencing involves real-time sequencing of single DNA molecules without needing to amplify DNA using PCR. Chip-based sequencing eliminates the need for expensive reagents and uses relatively inexpensive equipment, further lowering costs significantly, with increasing sequencing throughput speeds. Current gene sequencing technologies frequently require working with short snippets of DNA. These must be processed by large sequencers in a laboratory, and may take days to completion. 'Nanopore technology', the revolutionary advancement that the world stays tuned to will accelerate the genomic revolution. The excitement of nanopore DNA sequencing is in creation of 'tricorder-like' devices for detecting pathogens or diagnosing genetic disorders rapidly and on-the-spot, and may result in 'Point-of-Care' diagnostics for patients.

Besides, the human genome, one of the most compelling genetic mapping project is unraveling the genetic code of various cancers. Sequencing of cancer genomes allows scientists and doctors to discover gene mutations that contribute to cancer, potentially leading to better detection methods and treatments. The diagnosis and personalized treatment of cancer patients play a major role because the underlying cancer-causing mutations can vary greatly between tumors of different tissue and cell types, and between individuals with the same tumor. These genetic characteristics may have a huge

impact on the efficacy of anti-cancer drugs. Information gleaned through whole-genome sequencing has reclassified and stratified cancers based on the genetic makeup rather than only TNM classification of cancers and the location in the body. The system has resulted in a paradigm shift in cancer therapy for oncologists, and concurrent evaluation of the potential benefits of personalized cancer therapy. Besides, cost-effective approaches to whole exome (coding regions of the genome) sequencing available since 2009 (Maher, 2009), has been useful in predisposition studies in several cancer types (Jones *et al*., 2009) leading to 'Predictive Diagnosis' indicating critical importance of individual genomic constitution as a high risk factor.

'Deep Digital Sequencing' developed at The Genome Institute, Washington, USA, is commonly used to examine mutations in patients' tumor tissue samples, repeated 1000 times or more, generating frequency of the mutation. The data indicates evolution of cancer cells and molecular pathology of progression of the cancer (Maher, 2012). As cancer evolves, tumors acquire new mutations retaining the original cluster of mutations resulting in converting the normal cell to a malignant cell. The authors suggested that drugs targeted to genetic changes that occur early in the course of cancer may be more effective. On the other hand, drugs targeted to mutations observed exclusively in later-evolving cancer cells, may not have much effect on the disease and may not kill all the tumor cells. Sequencing is revolutionizing medical science and has the potential to serve as a powerful and cost-effective diagnostic tool in the management of cancer.

The Genomics Opportunity

Next-generation sequencing technologies have been and continue to be deployed in clinical laboratories, enabling rapid 'Bench to Bedside' transformations in 'Molecular Medicine'. As a reference point it is worthwhile to remember that the first complete cancer genome sequenced was that of acute myeloid leukemia (AML) cells, a severe form of cancer that initiates in the bone marrow. The AML genome was sequenced by creating single-read libraries from several micrograms of DNA, as replicate library-preps. Each of these was sequenced on 98 runs to generate 6 billion single-end 32bp sequencing reads. At 3 days per lane (a year on a Genome Analyser II, Illumina) and approximately USD 640 per lane in consumables, culminated in a final cost of USD 500,000. Today the same genome can be analyzed using 500 ng of DNA in a PCR-free library prep and run on one HiSeq X Ten (Illumina) to generate 375M PE1 50bp reads in 3 days for USD 1000. The masked and hidden costs will need to be added. However, the trend is clear, with whole genome sequencing 100 times faster and 500 times cheaper, thus making it useful for clinical

analysis (Core Genomics, 2014).

The advances in software development for molecular sequence analysis makes it feasible to analyze the vast terabytes of data generated by sequencing the genome, as 'big data' continue to aid the genomics revolution. The science has resulted in several startups flourishing with a torrent of venture capital dollars poured into the powerful 'genome interpretation' and 'data analytical' space. This in turn has led to understanding the clinical significance of genomic data to doctors and patients, thus affecting the most valuable stakeholder in the cycle, the patient.

The DNA sequencing market has expanded consistently to 18% a year and is expected to reach nearly USD 7 billion in 2016 (BCC Research, 2012) with cost price making it affordable, available and accessible to the ultimate consumers.

Genomics Research: Controversies

Study reported by Roberts *et al*. (2012) on 53,666 identical twins in cancer registries from the United States, Sweden, Finland, Denmark and Norway, to gauge the predictive capacity of personal genome sequencing clearly emphasizes that prediction will remain probabilistic and not deterministic as behavior, environment and random events may often tip the ability to be predictive with certainty (Kolata, 2012). Thus, today sequencing is frequently used to better understand the mechanistic aspects of diseases and preempt better therapies.

The need of the hour is to build databases of known disease-causing genetic mutations, robust sequencing and interpretation methodologies to validate cause-and-effect relationships between genes, behavior, environment and disease. Repeat and reproducible mutation data post sequencing large and varied populations will be reflected in routine clinical applications of the robust genomic information.

Promising Better Health

The promise of personal genomics is here to stay, with a major role in better health with 'Personalized Medicine', risk information for several diseases including cancers, diabetes and heart diseases; potential of individuals to metabolize drugs and the need for drugs to be personalized, not only for patient treatment but for identification of variants in carrier status in several diseases. The testing should be available and affordable to all. The personal genomic data will make it feasible for each individual to actively participate in shaping their health profiles and bettering health score cards. The promise of personalized medicine will have to be weighed against the challenges posed by the technological, financial and ethical limitations.

It is a game with many stakeholders: scientists, entrepreneurs, doctors, policy makers, investors and the most important stakeholder – the patient. We must tread with caution and rule with ethics.

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Stem Cells and Extra Cellular Matrices: Applications in Tissue Engineering

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The ability to artificially simulate the 'mechanical' niche, broadly termed as Extracellular Matrix (ECM), of the bone marrow determines success for stem cell growth, architectural organization and differentiation viz. tissue engineering. The advent of various natural and synthetic polymers has greatly influenced tissue engineering. The focus of the review is on various artificial niche simulations, ECM, scaffolds such as hydrogels, electrospun nano and micro fibers, bone-strengthening scaffolds and tissue infills. The utility of the ECMs in the treatment of various medical conditions including bone and cartilage tissues, nervous tissues, spinal cord and tendon tissues as well as wound healing, along with the ability of some ECMs in entrapment of elusive cell secretomes will be discussed. The future of tissue engineering has indeed got a new lease of life with polymer scaffolds and it is feasible that certain goals, thought of impossible so far, may become possible.

INTRODUCTION

Stem cells, or for that matter all cells, for formation of viable and functional tissues, require interaction with their specific niche. The niches comprise the biochemical niche, including, soluble factors, cytokines, chemokines, growth factors and several other factors. Further, the mechanical niche, the acellular compartment, provide scaffold for the biochemical niche. In a natural environment, both these niches together play a crucial role in cell growth, differentiation and fate determination, besides a very critical role in functional organ/organelle formation. A major hurdle in the area of tissue engineering is to understand and simulate the complex niches. The primary hurdle is creating a three dimensional (3D) atmosphere for cell growth, which will allow not only mimicking tissue architecture, but also creating a gradient of biochemical components in cell–cell interactions.

Our ability to artificially simulate this complex and the co-ordinated/regulated environment will be a major leap in ability to understand and thereby direct stem cell fate, propelling the cells into targeted functional tissue formation, the basic goal of tissue engineering.

The current mini-review will focus on the

Key words: Stem cell, ECM, cell secretome, scaffolds.
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mechanical niche component, broadly termed Extracellular Matrix (ECM) component to simulate the natural tissue composition. The understanding of the biochemical fraction of the niche and modus of choosing a material close to the natural niche are vast topics, and thus not dealt with here.

The advent of biocompatible polymers has enhanced the ability to perform grafting, implanting, delivery and substitution of non-functional biological tissue with function reinstating artificial options. These are fast emerging potential alternatives to autografts and allografts, in short supply and carry risks of disease transmission. The scaffolds are used to engineer various soft connective tissues such as skin, ligament, muscle and tendon, as well as vascular and neural tissues. And for advanced cell therapies, the ECMs aid in long-term cell culture in a 3D system, enhance cellular propagation and act as an efficient system for targeted cellular delivery.

Scaffolds

A large part of what can be achieved in tissue engineering is dependent on the types and functional abilities of the various extracellular matrices/scaffolds available. A multitude of scaffolds are currently available for cellular growth, cellular/non-cellular delivery, regeneration of damaged tissue and replacement of degenerated tissue. Many more are being added to the list every day.

The currently available scaffolds fall largely into two broad categories, natural and synthetic; subcategorized into degradable and non-degradable (Dandayuthapani *et al*., 2011). These properties largely depend on the composition, structure and arrangement of the constituent macromolecules, broadly characterized into ceramics, glasses, polymers and several others. Of these, natural and some biodegradable or non-biodegradable polymers are most commonly preferred for tissue engineering purposes, referred to as 'biomaterials'. Some of the naturally occurring polymers are silk, collagen, gelatin, fibrinogen, elastin, keratin, actin and myosin. Naturally occurring polysaccharides such as cellulose, amylose, dextran, chitin, and glycosamino glycans are most favoured for preparation of scaffolds/matrices due to the high levels of biocompatibility (Ratner *et al*., 2004).

Synthetic materials often mimic the physicochemical and mechanical properties of biological tissues, thus enhancing the ability to stand-in for and repair damage to functional tissue. Besides, synthetic polymers are highly valued for the ability to manipulate porosity, tensile strength, degradation time and mechanical characteristics. Additionally, reproducibility, mass production, structural uniformity and long shelf life render them cost effective (Gunatillake *et al*., 2006). Some of the commonly used polymers such as polylactic acid (PLA), polyglycolic acid (PGA), polylactide-*co*-glycolide (PLGA) and

polyhydroxyalkanoate (PHA) copolymers are most widely used polymers for tissue engineering (Chen *et al*., 2002; Ma, 2004). Hydrogel scaffolds are important as also array of polymeric scaffolds/matrices available to tissue engineers. Some of the natural hydrogels are collagen, fibrin, alginate, chitosan; while the synthetic counterparts include PLA and perfluoroalkoxy (PFA) derived polymers, poly(ethylene glycol) (PEG) derivatives and poly(vinyl alcohol) (PVA) (Behravesh *et al*., 2003; Bryant *et al*., 2004; Eyrich *et al*.,2007; Kim *et al*., 2004; Kong *et al*., 2003; Schmedlen *et al*., 2002; Solchaga *et al*., 2002; Suh *et al*., 2000; Wallace *et al*., 2003). Recently, our group has successfully demonstrated the use of Puramatrix hydrogel (Becton Dickinson, New Jersy, USA) for creation of a 3D equivalent of bone marrow (BM) niche *in vitro* (Sharma *et al*., 2012).

Of the many classes of synthetic materials used, polymeric composites are fast evolving as in demand scaffold materials, to mimic ECM-like environment. Consequently, these serve as cell propagation sites as well as cellular delivery modules. These also act as the mechanical component of the stem cell niche, thereby contributing actively to tissue formation.

The fabrication of successful 3D scaffolds is a complex phenomenon and involves special attention to factors such as macro/ microstructure, interconnectivity, surface charge and area, porosity and pore size, biocompatibility and mechanical strength. The ECMs most amenable to these functions are the electrospun matrices. These electrospun matrices/scaffolds allow flexibility of scaffold formation in the micro and nanometer range. The advent of 3D scaffolds that mimic the nano-architecture of biological tissues has opened up a host of avenues and possibilities in tissue engineering (Vasita *et al*., 2006). The mechanical properties and wide range of degradation patterns available for polymeric scaffolds are of great importance in the quest for nano-tissue engineering scaffolds/devices (Sokolsky-Papkov *et al*., 2007). One of these nanodevices is the electrospun nanofibre matrix, which shows great morphological similarities to various biological extracellular matrices. These are characterized by continuous fibres, high surface to volume ratio, high porosity and manually variable poresize. Electrospun nanofibres may be tagged with various biocompatible/bioactive molecules, thereby increasing the possibilities of cellular adherence and growth. This enables supply of necessary chemical cues for growth of specific cell types. The tensile strength of the scaffolds allows use in cell delivery in *in vivo* experiments (Kumbhar *et al*., 2008). Most interestingly, the tensile strength of the scaffolds are remarkably similar to skin and marginally lower than human cartilage, demonstrating that nanofibre scaffolds are

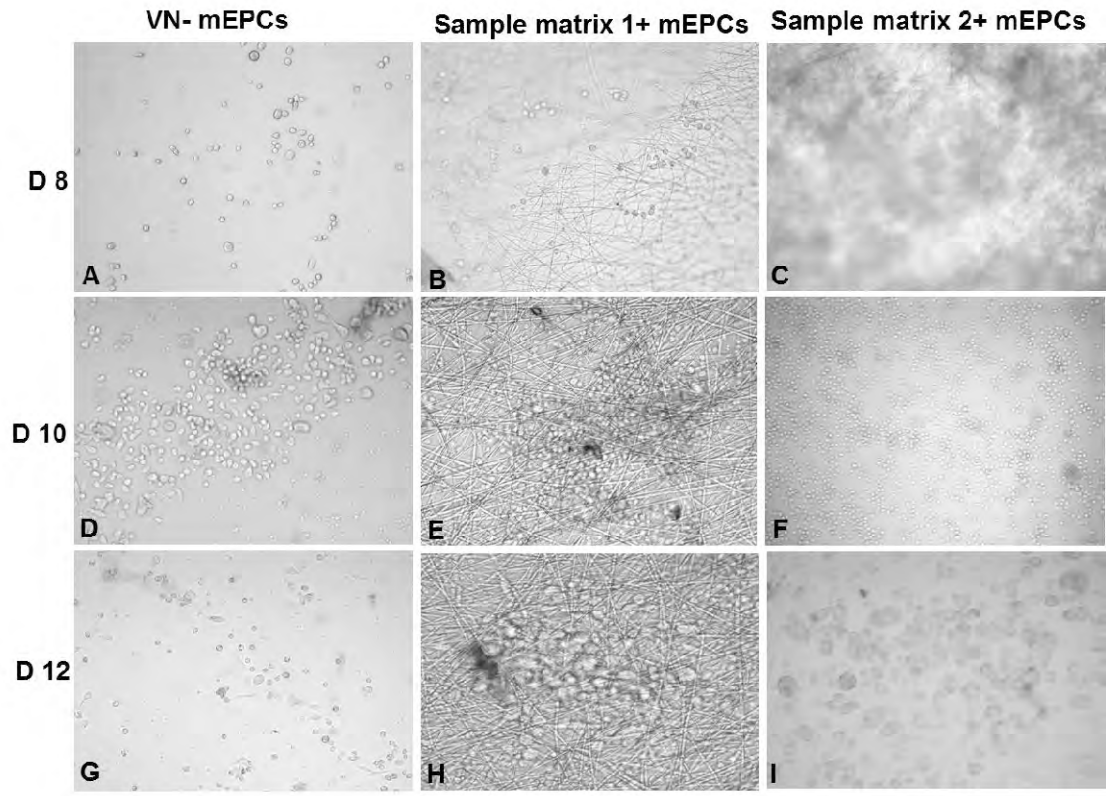

Figure 1: Light microscope image depicts two preparations of electrospun nanofiber matrices (3D systems) supporting varying degrees of endothelial progenitor cell (EPC) growth from day 8 to 10. Vitronectin is the standard 2D control, which also supports EPC growth, albeit to a markedly lesser extent.

candidates for implantation or for regeneration of cartilages (Fischer *et al*., 2012; Shin *et al*., 2006).

The use of these biofriendly polymeric materials has added to the vistas for the types and extent of tissues regenerated, particularly for stem cells, given their higher requirement for niche regulated support. The 3D architecture of ECMs/scaffolds allows enhanced cell growth as well as tissue like intercellular interactions. The thickness of the matrix component influences cell–cell dynamics and eventual tissue application. As represented in the microphotograph, sample matrix 1 is thinner than sample matrix 2 (details withheld so as to not compromise patent filing) and consequently shows lower cellular growth from d8 to d10 (Fig. 1). A benefit to a thinner matrix enhances the visualization potential.

In context, it is evident that certain biological symptoms and disorders have benefited more than others due to the usage of nanofibrous and other ECMs/scaffold induced tissue applications. Several of the disorders are related to skin, bone, cartilage, liver, heart valves, arteries, bladders, pancreas, nerves, tendons, spinal cord, corneas and other soft

tissues (Boyan *et al*., 1999; Diedwardo *et al*., 1999; Eaglstein *et al*., 1998; Germain *et al*., 1999; Mayer *et al*., 1997; 2000; Mohammad *et al*., 2000; Oberpenning *et al*., 1999; Tziampazis *et al*., 1995).

Bone

Osteoporosis is induced by impaired balance between the activities of cellular constituents of the bone, osteoblasts and osteoclasts. ECMs facilitate formation of osteoblasts from non-osteo lineage stem cells, such as mesenchymal stem cells (MSCs). Yoshimoto *et al*. (2003) successfully cultured and expanded MSCs on polycaprolactone (PCL) scaffolds and propelled them into osteogenic lineage under dynamic culture conditions for four weeks. Interestingly, cell-embedded matrices maintained the size and shape of the original scaffold (Yoshiomoto *et al*., 2003). Since osteoporosis make bones fragile, bone grafts are important. Mineralized polymeric nanofibrous composites have been successfully employed as materials for bone grafts (Ngiam *et al*., 2009*)*. Although bone formation is a crucial step in regeneration, it alone does not suffice for larger bones, such as femur performing crucial weight bearing functions. Complete regeneration of these bones has been a hurdle. However, applications of ECMS/scaffold techniques have made this feasible. For the purpose of load-bearing tissue engineering, a novel biodegradable nanocomposite porous scaffold comprising a b-tricalcium phosphate (b-TCP) matrix and hydroxyl apatite nanofibers has been developed by a method combining gel casting and polymer, resulting in bone formation with enhanced capacity for load bearing (Ramay *et al*., 2004). Recently, a new composite material consisting of mesoporous bioactive glass (MBG) and concentrated alginate pastes were used for fabrication of hierarchical scaffolds by 3D plotting. This scaffold structure contains well ordered nano channels, micropores and controllable macropores beneficial for bone tissue engineering applications and drug delivery (Luo *et al*., 2013).

Sponge techniques

Apart from the usual type of scaffolds, natural polymers such as silk have been tested for their bone-building ability. Studies on the effect of primary or multiple silk coating revealed efficacy of these natural polymers in improving mechanical and biological properties of biphasic calcium phosphate (BCP) scaffolds, including *in vitro* evaluation of the osteogenic response of human MSCs (hMSCs) on the coated scaffolds. The multiple silk coating proved to be a simple, yet an effective technique for reinforcement. This could also be applied to other types of ceramic scaffolds with similar microstructure to improve osteogenic outcomes (Bogush *et al*., 2009; Li *et al*., 2013). With current developments in the ECM technology, it has

become possible to integrate ECM components with non-degradable synthetic components, including beads. This technological advance is useful in bone morphogenesis. hMSCs entrapped in alginate hydrogel loaded with ECM coated beads, contributed to enhanced bone formation *in vitro*, indicating that engineered ECM may be employed in a minimally invasive manner to direct formation of bony tissue (Bhat *et al*., 2013). Current techniques have also facilitated slow release of bone formation related proteins, such as bone morphology protein-2 (BMP-2), by complexing them with various ECM components such as dermatan sulphate (DS), hyaluronic acid (HA) hydrogels. *In vivo* studies on rats demonstrated that HA-hydrogel delivered BMP-2 precomplexed with glycosamine glycans (GAGs) induced twice the amount of bone formation compared to controls (Kisiel *et al*., 2013).

Vascular engineering

The idea that ECM may be able to influence microvasculature of endothelial cells and promote angiogenesis is not a new one. Feng *et al*. (1999) demonstrated that ECM environment could regulate human dermal microvasculature and promote endothelial cells into higher microvessel formation (Feng *et al*., 1999). The advent of nano-fiber technology has amply benefited the field of blood vessel formation, vascular grafts etc.

Currently, different types of stem cells are used for formation of blood vessels including MSCs and endothelial progenitor stem cells (EPCs). Hashi *et al*. (2007) used nanofibrous grafts for regeneration of vascular grafts and successfully employed the antithrombogenic properties of BM-MSCs for tissue vascularization. Coronary artery smooth muscle cells, also capable of forming blood vessels, have been successfully employed for long term vascularization using poly-L-lactic-co-ε-caprolactone nanofibrous scaffolds (Dong *et al*., 2008). Cell numbers often demarcate the efficacy of an available graft; thus increasing the need for 3D scaffolds to enhance cellularization (Williamson *et al*., 2006). Mun *et al*. (2012) have used 3D electrospun nanofiber poly-L-lactic acid (PLLA) matrices for small diameter vascular grafts, thereby enhancing functionality of the graft (Mun *et al*., 2012). The poly-caprolactone-polyurethane (PCL-PU) composite scaffold was developed by wet spinning PCL fibres which form the luminal surface, then electro-spinning porous PU onto the back of the PCL fibres to form the vessel wall substitute. This was successfully used as a device for small diameter vascular grafts and showed high capability for endothelial cell attachment and proliferation to form a monolayer with strong platelet/endothelial cell adhesion molecule-1 (PECAM-1) expression and cobblestone morphology (Hau-Min *et al*., 2013).

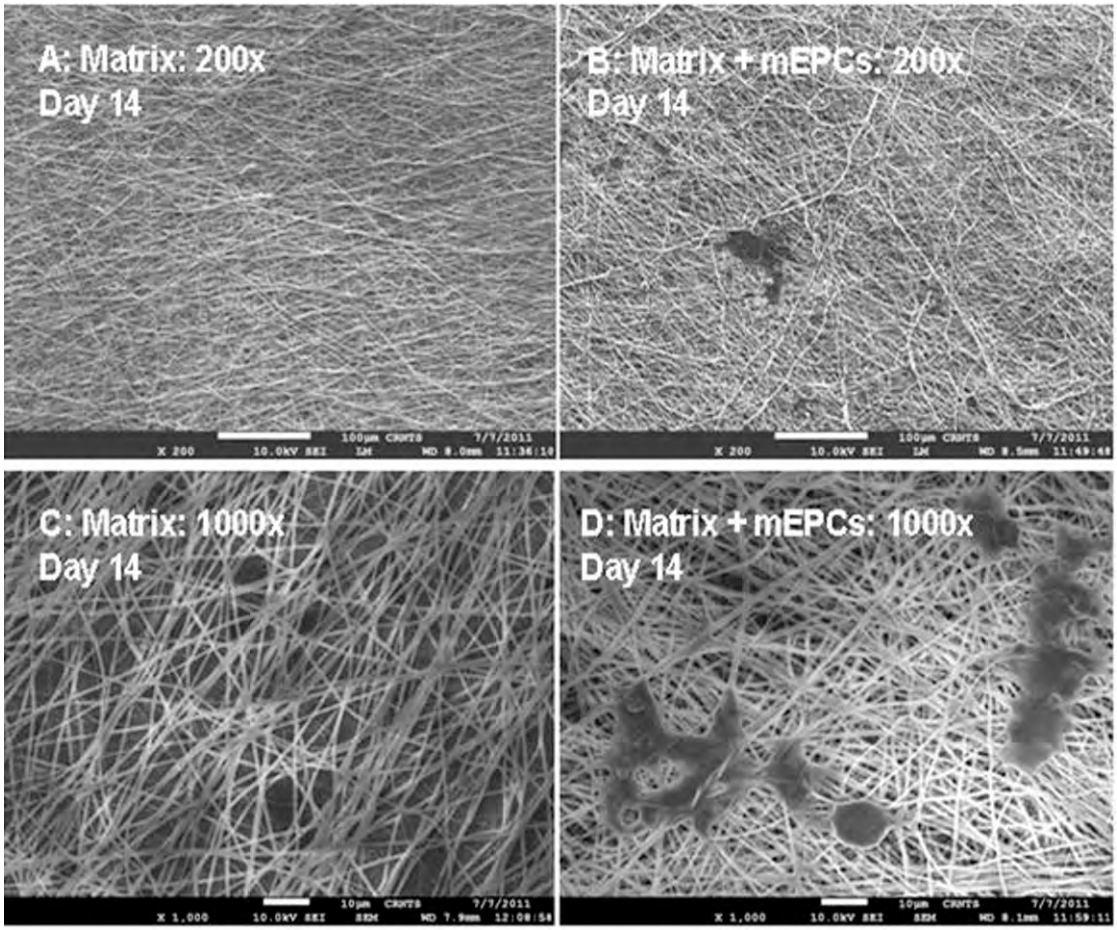

Figure 2: Scanning electron microscopy image depicts PCG matrix supporting murine EPCs for a long term culture, while maintaining cellular morphology. Images of PCG matrix without EPCs (A) and with EPCs (B) are illustrated (Magnification 200x). Images of PCG matrix without (C) and with (D) m-BM-EPCs at day 14 in culture (magnification 1000x).

Nerve, tendon and spinal cord tissue engineering

ECMs/scaffolds have benefitted the field of nerve tissue engineering. Several different types of polymers have made their mark for development of nervous tissues including hyaluronan-gelatin, etc. Yang *et al*. (2004) developed a porous polymeric nanofibrous scaffold using a biodegradable polymer, PLLA, for *in vitro* culture of nerve cells. Since then PLLA has been widely used in tissue engineering for a variety of purposes besides nerve tissue engineering. Similar polymers and derivatives, such as microspheres, have also been deployed with advantage. Polyphosphoester miscrospheres or polymer bound natural biomaterials, have been used with success for sustained release of biologically active nerve growth factors leading to enhanced growth of nerve cells (Sun *et al*., 2009; Xu *et al*., 2002). Tendon neogenesis has also benefited from

development of these scaffolds (Xu *et al*., 2013). Spinal cord engineering has benefited greatly by hydrogel type of tissue infills, which cover the sheath and eventually contribute to spinal cord regeneration (Macaya *et al*., 2012).

Wound healing

The basic problem in using stem cells for wound healing applications, bandage style, is the cell loss due to flow away mechanisms, reducing efficacy of the transplanted cells. For this purpose, a matrix that can function both as a cell growth substrate and cell delivery scaffold will be most efficacious. The technique of electrospinning various polymers into nano/microfibrous scaffolds has revolutionized the field of wound repair using stem cells. In an interesting study, human adipose tissue derived stem cells were seeded onto a silk–fibrin–chitosan scaffold. The cells not only enhanced wound healing in a soft tissue injury mouse model, but also demonstrated differentiation into various lineages linked to wound healing, such as fibrovascular endothelial and epithelial cells in the restored tissue (Altman *et al*., 2009). Studies have also revealed that self assembling peptide nanofiber scaffolds accelerate wound healing in a bioengineered Human Skin Equivalent (HSE) tissue model that enabled wound re-epithelialization to be monitored in a tissue that recapitulates molecular and cellular mechanisms of repair in human skin (Lahiji *et al*., 2000). Similar studies showed successful results in burn wounds (Meteroja *et al*., 2013). In our laboratory polycaprolactone-gelatin (PCG) electrospun nanofibrous matrix is in use for long term and enhanced EPC culture, as a 'ready-to-use' EPC delivery scaffold for treatment of diabetes induced impaired wound healing (Fig. 2). The application of the matrix embedded cells enhanced the rate of EPC growth about four times as the controls; while application of the PCG embedded EPC patch onto wound sites in diabetic mice, enhanced wound healing rate significantly, indicating the tremendous potential of such treatments for similar medical conditions (Fukuda *et al*., 2006).

ECM assisted co-culture systems

Cell co-culture systems are used in several fields of biomedical sciences. Consequently, advances in the techniques on the interface of tissue and biological engineering contributed to several types of tissue culture systems requiring co-culture, or multi-culture of various cell types. A simple interface system using chitosan was devised as early as 2000, for human osteoblasts and chrondrocytes (Nagata *et al*., 2002). Cartilage tissue engineering is a complex subject. A co-culture system comprising MSCs and chondrocytes has proved promising for development of other types of cells. Its benefits were recently harvested for creation of hypoxia, deemed to be beneficial for cartilage development

(Schneider *et al*., 2008). In an ingenious approach, Fukuda *et al*. (2006) created micro patterned cell co-cultures using two ECMs deposited one on top of the other. The system demonstrated the potential benefit of growing more than one type of cell(s) (Meng *et al*., 2009). Collagen matrices have been known to retard, and perhaps increase overall longevity of rat pancreatic islets of Langerhans (Bakota *et al*., 2011). Our recent data (unpublished data, personal communication) indicated successful culture of three cell types, in varying proportions using a simple, electrospun nanofibrous matrix. The results implied promise of harvesting and harnessing the properties of elusive secretomes (unpublished data, personal communication). This approach emphasizes importance of multiple cell culture engineering over simple ECM regulated cultures. The approach may reveal new routes of stem cell and primary cell co-cultures.

Cellular secretomes

Recently, it has been demonstrated that not only the cells, but the cellular secretomes can be harnessed for therapeutic purposes. Recently, several groups have harnessed the MSC secretome for treatment of cardio-vascular disease (Wang *et al*., 2011). Several other studies follow similar patterns. Taking a lead from this secretome dependent therapeutic approach, Bakota *et al*. (2011) devised an injectable multi domain peptide nanofiber hydrogel as a delivery agent for stem cell secretome. At a concentration of 1% by weight, this peptide forms extensive nanofibrous network, resulting in a physically crosslinked viscoelastic hydrogel. The hydrogel undergoes shear thinning and quickly recovers 100% of its elastic modulus when the shearing force is released, making it ideal for use as an injectable material (Kanitkar *et al*., 2013). The group also used secretome pre-conditioned peptide nanofibers for renal protection following acute kidney injury (Ranganath *et al*., 2012). Contextually, harvesting the cell secretome is a tedious task typically involving collection of conditioned media and enrichment of active components, which may result in loss of several labile molecules like proteins and peptides. The nanofibrous matrices with small pore sizes may be employed for entrapment and easy harvesting of these cell secretomes with hydrogel-like consistency (unpublished data, personal communication).

The cellular secretomes may possibly mimic the exact biochemical component of the stem cell niche and hence special efforts should be directed at understanding the composition and functionality of the 'secretome'. Indirectly, the artificial mechanical component may allow us to 'trap' and analyse the biochemical component.

The current overview highlights the applications of various ECM/scaffold induced regeneration by promoting cell growth and/or

permit cell delivery. The examples and citations give an idea of the extensive application in the field of disease biology and the benefits accrued. The resourcefulness and efforts of the scientific community in the field has created a range of scaffolds, with respect to materials, thickness, pore size, degradability, shapes such as sheets, cylinders, fibres, micro/mega spheres etc., to choose from depending on the specific application. The future of tissue engineering has indeed got a new impetus with polymer scaffolds and multiplied the implications in biomedical applications.

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CONFLICT OF INTEREST

The authors claim no conflict of interest.

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Molecular Basis of Reprogramming: Modulation by microRNAs

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Induced pluripotent stem cells (iPSCs) have opened up a new avenue for customized regenerative medicine. iPSCs can be generated by forced expression of transcription factors, Oct4, Sox2, c-Myc and Klf4. Although reprogramming techniques are well documented, one of the major concerns has been the poor efficiency of reprogramming. The reprogramming efficiency can be enhanced using various chemical compounds and vector systems. However, low reprogramming efficiencies and use of viral based vector systems limit clinical application of iPSCs. microRNAs (miRNAs) are extensively studied due to their critical role in numerous biological activities like cell cycle regulation, growth control and apoptosis. Discovery of embryonic stem cell (ESC) specific unique miRNAs, encouraged researchers to study contribution of miRNAs towards embryonic stem cell development, differentiation and somatic cell reprogramming (SCR). Depletion of mouse embryonic fibroblast (MEF) enriched miRNAs like miR-29a, miR-21 and let-7, are necessary to enhance reprogramming. Furthermore, up regulation of miR-200, miR-106a/b miR-120, miR-93 miR-301, miR-17, miR-721, miR-29b is required for mesenchymal-to-epithelial transition (MET), a critical initial event during the generation of iPSCs from fibroblasts. The expression of embryonic stem cell specific miRNAs like miR-290/miR-302 cluster, miR-367/miR372 is crucial to maintain pluripotent status of iPSCs. In this review, we discuss contribution of miRNAs to generation of iPSCs, their defined role in maintenance of pluripotent state, transcriptional regulatory networks and epigenetic factors to modulate reprogramming.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) are a type of adult stem cells genetically reprogrammed to an embryonic stem cell (ESC) like state. Human ESCs established in 1998 (Thomson *et al*., 1998) are considered promising sources for cell transplantation. However, use of human ESCs has several ethical constraints that hinder its application in regenerative medicine. Moreover, the use of human ESCs for clinical application must overcome barriers such as immune rejection, tissue regeneration and teratoma formation. An alternative to overcome these hurdles is to reprogram a patient's own somatic cells to iPSCs (Takahashi and Yamanaka, 2006), a Nobel prize winning contribution pioneered by Yamanaka and co-workers. The authors demonstrated direct reprogramming of mouse (Takahashi and Yamanaka, 2006) and human

Key words: miRNAs, reprogramming, iPSCs, stem cells, pluripotency.
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fibroblasts (Takahashi *et al*., 2007) to a pluripotent state, generating induced pluripotent stem cells (iPSCs). The generation of iPSCs revolutionized regenerative medicine research by introducing a method to supply an adequate number of patient-specific pluripotent cells for therapeutic transplantation, thus obviating the need to use human embryos.

The generation of iPSCs by Yamanaka and colleagues was achieved by overexpressing important pluripotent transcription factors, initially in mouse (Takahashi and Yamanaka, 2006), followed by human fibroblasts using retroviral system. The factor comprised Sex-determining region Y HMG box 2 (Sox2), Krüppellike factor 4 (Klf4), Octamer binding transcription factor 4 (Oct4), and myc myelocytomatosis viral oncogene homolog (c-Myc), are referred as the 'Yamanaka factors' (OSKM) (Takahashi *et al*., 2007). Briefly, Oct-4 and Sox-2 are transcription factors, for maintaining the pluripotency of stem cells (Chen and Daley, 2008). c-Myc plays a major role in early reprogramming stages and enhances generation of partially reprogrammed cells (Koche *et al*., 2011; Schmidt and Plath, 2012). Direct interaction of Klf4 with pluripotent genes, Oct4 and Sox2, is critical for somatic reprogramming (Wei *et al*., 2009). Thomson and colleagues used another combination of reprogramming factors viz., Oct-4, Sox-2, Nanog (homeobox protein Nanog) and Lin-28 (mRNA binding protein expressed in embryonic stem cells) (Yu *et al*., 2007). Such genome integrating viral vectors produce mutagenic lesions that are potentially tumorigenic or influence differentiation potential. Therefore, several approaches have been developed to generate novel, non-integrating methods for iPSC generation. Recent studies have indicated that iPSCs can be obtained with virus-free, expression plasmid or PiggyBac transposons (Jia *et al*., 2010; Kaji *et al*., 2009; Malik and Rao, 2013; Narsinh *et al*., 2011; Woltjen *et al*., 2009). Gonzalez and colleagues (2010), generated iPSCs from mouse embryonic fibroblasts using polycistronic construct co-expressing Oct-4, Sox-2, Kfl4 and c-Myc. However, several rounds of transfection were necessary to maintain expression of transgene at the level required to generate iPSCs (Gonzalez *et al*., 2009). The reprogramming efficiency was significantly lower than using the viral vector systems. Subsequently, modified expression plasmid based technique used a polycistronic non-viral minicircle plasmid vector to genetically reprogram human adult adipose derived stem cells (Jia *et al.,* 2010). The integration free human iPSCs generated by this technique indicated reprogramming efficiency of approximately 0.005%, much lower than integrating viral based method. Further, introduced sequences employed in these approaches, could integrate into the genome as DNA constructs. The safety issue of iPSCs led to the use of protein based

methods for generation of pluripotent stem cells. Zhou *et al*. (2009), for the first time, reported generation of protein induced pluripotent stem cells (pi-PSCs) from mouse embryonic fibroblasts using recombinant cell penetrating reprogramming proteins. A protein transduction domain, poly-arginine fused to the c-terminus of Yamanaka factors (OSKM) in order to obtain recombinant proteins that can penetrate across the plasma membrane of somatic cells. The approach significantly improved reprogramming efficiency (Zhou *et al*., 2009). However, the procedure involved is technically challenging (Kim *et al*., 2009a; Wang *et al*., 2013). Embryonic stem cells possess a unique set of microRNAs (miRNAs) (Houbaviy *et al*., 2003; Suh *et al*., 2004), with a crucial role in embryonic development and absence of the miRNAs impede cell proliferation and differentiation (Kanellopoulou *et al*., 2005; Murchison *et al*., 2005). Thus, miRNA 302/367 cluster is highly expressed in ESCs and downregulated in cell differentiation, encouraging study of the role of miRNA 302/367 cluster in reprogramming (Lin *et al*., 2011; Miyoshi *et al*., 2011; Subramanyam *et al*., 2011; Zhang *et al*., 2013). Numerous miRNA-mediated iPSCs lines have been derived from mouse fibroblast, human skin and dermal fibroblasts using only miR302 cluster or combination of numerous ESCs specific miRNAs. A key feature in ensuring effective reprogramming is epigenetic remodeling. The crucial role of miRNAs in regulating SCR and various approaches using miRNAs for reprogramming are discussed.

Biogenesis of miRNAs

miRNAs belong to a class of endogenous, single stranded, small non-coding RNAs of 19–22 nucleotides, derived from a 70-nucleotide precursor (Bartel, 2004; Lakshmipathy and Hart, 2008). miRNAs regulate expression of target genes by at least two mechanisms – translational inhibition or by promoting degradation of mRNAs (Krol *et al*., 2010). miRNAs were initially discovered in *Caenorhabditis elegans* (Lee and Ambros, 2001) and subsequently studied in green algae, viruses, plants and mammalian cells (Griffiths-Jones *et al*., 2008; Odling-Smee *et al*., 2007; Pentimalli *et al*., 2007). miRNAs act as key regulators of processes including developmental timing, patterning, growth control, apoptosis and tumorigenesis (Choi *et al*., 2013; Farazi *et al*., 2011; Gangaraju and Lin, 2009; Ivey and Srivastava, 2010; Lima *et al*., 2011; Subramanyam and Blelloch, 2011; Zhao and Srivastava, 2007). miRNAs play a crucial role in maintenance of stem cell pluripotency (Jia *et al*., 2013; Heinrich and Dimmeler, 2012) and critically regulate stem cell fate decisions, including self-renewal and differentiation into specific lineages (Guo *et al*., 2011).

In mammals, the biogenesis of miRNAs and their mechanism of action have been well

characterized (Carthew and Sontheimer, 2009; Huntzinger and Izaurralde, 2011). The miRNA canonical processing pathway, utilizes a 70-nucleotide primary miRNA (pri-miRNA) transcript which gets processed into stem loop precursor miRNA (pre-miRNA) by Drosha-DGCR8 enzyme complex in the nucleus (Carthew and Sontheimer, 2009). In the noncanonical pathway, pre-miRNA is generated from small introns also called mirtrons by alternative splicing and a debranching enzyme that generates a short hairpin, for processing by Dicer. This pathway circumvents requirement of the Drosha-DGCR8 complex that is required in the canonical pathway. In both cases, the pre-miRNA hairpins are translocated in the cytoplasm by exportin 5 where they are processed to mature miRNAs. In the cytoplasm, the ribonuclease type III, Dicer cleaves selectively the terminal loop of precursor to generate approximately 19–22 nucleotide mature miRNA/miRNA* duplex (Kim, 2005). Subsequent to Dicer processing, one of the two strands of the duplex, derived from both canonical and noncanonical pathways, is incorporated into the miRNA-inducing silencing complexes (miRISCs), through its interaction with one of the member of the argonaute (Ago) family. Ago class 2 protein is the only mammalian protein capable of directly cleaving the complementary target of mRNAs. Hence, miRISC silences the expression of target genes predominantly through a posttranscriptional repression, and the silencing of specific targets is dependent on a base-pairing interaction between the incorporated miRNA and the target (Krol *et al.,* 2010). It is postulated that approximately 1–5% of genes in animals encode miRNA and miRNAs target approximately 10–30% protein coding genes (Krol *et al.,* 2010).

The recent high throughput next generation massively sequencing (NGMS)

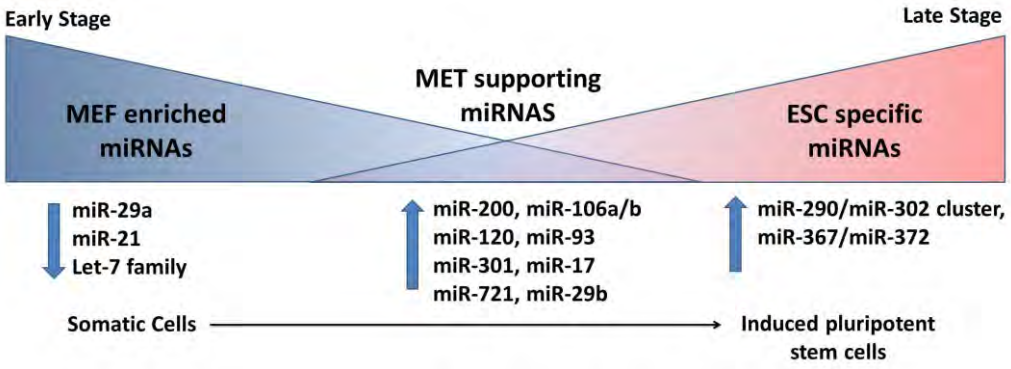

Figure 1: Expression of miRNAs during iPSCs generation. MEF enriched miRNAs downregulate at early stage. Simultaneously miRNAs that positively regulates MET and pluripotent state upregulate. Somatic cells in early stage and late stage of reprogramming show expression patterns of miRNAs that closely resemble somatic cells (fibroblasts) and embryonic stem cells, respectively. Abbreviations – MEFs: Mouse embryonic fibroblasts; MET: Mesenchymal-epithelial transition; ESCs: Embryonic stem cells; miRNA: microRNA.

technology has been used to identify numerous miRNAs. These technologies have modernized genomic research, allowing many mammalian miRNAs to be identified and deposited in miRBase (www.mirbase.org). Till date, 24,521 entries of hairpin precursor miRNAs, with 30,424 matured miRNA products in 206 species have been recognized and deposited in the public miRNA database miRBase (Release 20.0, June 2013). Amongst these, 2578 miRNAs are of human origin. A proper prediction and validation of miRNA targets is essential to understand function of miRNAs. Computational prediction identify that all genes are regulated by miRNAs and single miRNA can target several genes. The analysis of miRNA predicted targets is performed using different algorithms like, TargetScan, PicTar, miRanda. Furthermore, validation of predicted targets can be done by reporter assays for testing predicted functional miRNAs target sites.

miRNAs during initial stage of reprogramming

Early phase of reprogramming includes expression of miRNAs that inhibit apoptosis and enhance cell proliferation (Fig. 1). An elevated level of p53 in the initial stage of reprogramming reduces the overall iPSCs formation efficiency (Sarig *et al*., 2010; Tapia and Schoer, 2010). Moreover, one of the p53 target, cyclin-dependent kinase inhibitor p21, causes cell cycle arrest or favors apoptosis (Bodzak *et al*., 2008; Kawamura *et al*., 2009). An unrevealed crucial role of miR-138 in regulation of p53 pathway and promotion of iPSC generation was first reported by Dan and colleagues (Ye *et al*., 2012) (Fig. 2). Briefly, p53 is down regulated by miR-138 which in turn reduces expression of p21 and miR-34 during somatic cell reprogramming (Choi *et al*., 2011; Ye *et al*., 2012). miR-34 cluster (miR-34a,-34b,-34c) is a barrier to reprogramming as it reduces expression of pluripotent factors like Oct-4, Nanog (Choi *et al*., 2011; Ng *et al*. 2014). Ye *et al.* (2012) reported that, endogenous expression of Oct4 and Sox2 genes is relatively low and retroviral expression remains active in iPSCs generated by OSKM factors from p53-null cells. Moreover, ESC-like morphology cannot be maintained after passage five. Alternatively, in this study, the morphology of miRNA-mediated reprogrammed iPSCs (138-iPSCs), was similar to that of mouse ESCs and were maintained for more than 20 passages *in vitro*. Additionally it was reported that p53 binds to the miR-145 promoter and activates its expression (Sachdeva *et al*., 2009; Suh *et al*., 2011). miR-145 known to induce differentiation of ESCs by suppressing the expression of reprogramming factors, Oct4, Sox2 and Klf4. Hence, miR145-p53 axis is a roadblock to reprogramming (Liu *et al*., 2012; Xu *et al*., 2009) (Table 1 and Fig. 2).

miRNAs expressed in mouse embryonic fibroblasts (MEFs) interfere with

reprogramming efficiency (Melton *et al*., 2010). Depletion of MEFs enriched miR-29a and miR-21 result in an enhanced reprogramming efficiency mediated by regulation of ERK1/2 and p53 pathways (Yang *et al*., 2011; Yang and Rana, 2013). The depletion of miR-29a using inhibitors, decreased p53 protein levels by elevating p85α and CDC42 expression (Yang *et al.,* 2011), and depletion of miR-21, decreased ERK1/2 phosphorylation (Yang *et al.,* 2011). Further, c-Myc has shown to repress miR-29a and miR-21 to promote reprogramming (Yang *et al.,* 2011) (Table 1).

Let-7 family of miRNAs (Let-7a1, -a2, -a3, -b, -c, -d, -e, -f,1,-f2, -g, -i) are abundantly expressed in MEFs (Pasquinelli *et al*., 2000; Reinhart *et al*., 2000) (Fig. 1), leading to investigation of the role of let-7 family of miRNAs in reprogramming. The miRNAs are pluripotent silencing miRNAs as they inhibit expression of a number of pluripotent regulators, including Sall4, Lin-28b, Hmga2 and c-Myc, n-Myc (Kim *et al*., 2009b; Melton *et al*., 2010; Park *et al*., 2007; Rybak *et al*., 2008; Sampson *et al*., 2007). c-Myc inhibits expression of let-7 through Lin-28b transactivation and depletion of let-7 elevates reprogramming efficiency four fold with OSK reprogramming factors (Melton *et al.,* 2010). The let-7 family of miRNAs act as a barrier to reprogramming via expression of prodifferentiation genes including early growth response protein 1 (EGR1) (Worringer *et al*., 2013). The inhibition of let-7 with the OSK cocktail increases the reprogramming efficiency of human dermal fibroblasts (HDF) comparable to that with OSKM. Further let-7 inhibition augments OSK mediated reprogramming, at least in part through promoting LIN-41 expression. EGR1 mRNA is bound and negatively regulated by LIN-41 and blocks reprogramming. Together these findings delineate the role of a let-7-based pathway that counteracts the activity of reprogramming factors through promoting the expression of prodifferentiation genes (Chang *et al*., 2009; Worringer *et al.,* 2013) (Table 1).

c-Myc, one of the four reprogramming factors, induces expression of a number of miRNAs that favor initiation of the early transitional stage (Yang *et al.,* 2011; 2013). c-Myc induces repression of MEF enriched miRNA, miR-21 and miR-29a enhancing the early phase of reprogramming events (Yang *et al.,*, 2013). Furthermore, c-Myc alone can augment expression of miR-17`92 cluster, miR-106b`25 cluster and miR106a`363 cluster expressions (Li *et al*., 2011; Mendell, 2008). Recently, He and colleagues (2014), reported that miR 19a/b of cluster miR17`92 were significantly induced by c-Myc during initial stage, suggesting a crucial role of the miRNAs during somatic cell reprogramming. The enhancement of reprogramming by miR-19a/19b was mediated by repressing expression of tumor suppressor protein, phosphatase and tensin homolog (PTEN),

causing cell cycle arrest (Weng *et al*., 2001). These results suggest that cMyc-miR-19a/b-PTEN axis plays a crucial role in reprogramming human somatic cells. The approach circumvents the use of c-Myc, hence miR17`92 cluster can be used to reprogram somatic cells into iPSCs for clinical purpose (He *et al*., 2014) (Table 1).

miRNAs promote mesenchymal-to-epithelial transition

An early event during iPSC generation is mesenchymal-to-epithelial transition (MET). Factors that promote MET or inhibit epithelial-to-mesenchymal transition (EMT) help in reprogramming. A prominent observation in early days of reprogramming is the transformation into cluster of cells resembling epitheloid morphology. Inhibition of EMT occurs by suppression of transforming growth factor β (TGF-β) pathway (Li *et al*., 2010; Miyazono, 2009). miR-106a, miR-106b, miR-93 and miR-17 accelerate reprogramming by targeting TGF βII (Li *et al.,* 2011) (Fig. 1). Another family of miRNAs, miR-130, miR-301 and miR-721 enhanced mouse fibroblast reprogramming by reducing expression of homeobox transcription factor, Meox2 (or Gax) (Pfaff *et al*., 2011). Meox2 is associated TGFβ pathway (Valcourt *et al*., 2007) (Fig. 2). miR-200 downregulates expression of MET barriers including ZEB1 and ZEB2 (Burk *et al*., 2008; Gregory *et al*., 2008; Korpal *et al*., 2008) (Fig. 2). ZEB1 and ZEB2 (mesenchymal markers) are transcriptional repressors of E-Cadherin and master regulators of epithelial polarity (Bracken *et al*., 2008). Although it is now known that fibroblasts can be reprogrammed to an ES like state, the underlying mechanism is not clear. He *et al.* (2014) reported Oct-4 and Sox-2 positively regulate expression of miR-200, which in turn down-regulates mesenchymal marker ZEB2 through directly targeting the 3'UTR. ZEB2 is member of the ZFHX1 family of two-handed zinc finger/homeodomain proteins, initially shown as a binding partner of SMAD1 and SMAD2/3. Thus, miR-200s regulate expression of Sox-2/Oct-4 during iPSCs generation, and miR-200s/ZEB2 axis play crucial roles in Sox-2/Oct-4-initiated MET process during reprogramming (He *et al*., 2014). During iPSCs generation, a change in DNA methylation pattern is essential for epigenetic remodeling and the re-establishment of the ESCs-specific gene expression profile (Mikkelsen *et al*., 2008). In addition, DNA methylation leads to reactivation of epithelial specific markers in the MET process. The reactivation of imprinted regions, like *Dlk1-Dio3* locus, is essential for development of fully pluripotent iPSCs (Liu *et al*., 2010). DNA hyper-methylation leading to silencing of *Dlk1-Dio3* locus prevents cells from becoming fully pluripotent iPSCs (Liu *et al.,* 2010; Li *et al*., 2010). Hence, DNA methyl transferases

Table1: miRNAs: Regulators of induced pluripotency

microRNAs	Targets	Function in reprogramming	Reference
miR-34 cluster-miR-34a, miR34b, miR-34c	Oct-4, Nanog	Barriers	Choi *et al* 2011; He *et al*., 2007, Ye *et al.,* 2012
miR-145	P53/ Oct-4, Sox-2,Klf-4	Barrier	Liu *et al*. 2012; Xu *et al*., 2009
miR-29a	P53 pathway	Barrier	Yang *et al*., 2011
miR-21	ERK1/2 phosphorylation	Barrier	Yang *et al*., 2011
Let-7 family	Sall4, Lin-28b, Hmga2, c-Myc, N-Myc	Barrier	Kim *et al*., 2009b; Melton *et al.*, 2010; Park *et al.*, 2007 Rybak *et al*., 2008; Sampson *et al*., 2007
miR19a/miR19b	PTEN	Promoters	He *et al*., 2014
miR106a, miR106b, miR-93, miR-17	TGFβII	Promoters	Li *et al.*, 2011
miR-130,miR301, miR-721	Meox1	Promoters	Pfaff *et al.*, 2011
miR-200	ZEB1 and ZEB2	Promoter	He *et al*., 2014; Burk *et al*., 2008; Gregory *et al*., 2008; Korpal *et al*., 2008
miR-29b	Dnmt3a, Dnmt3b	Promoter	Guo*et al*., 2013
miR 302, miR372, miR-367	TGFβ, MECP2, MBD2,SMARCC2, NR2F2	Promoters	Hu *et al*., 2013; Subramanyam*et al*., 2011
Mouse: miR-291a-3p, 291b3p, 294, 295, 302a-d	cdkn1a,Rb1, Rb2	Promoters	Judson *et al*., 2009
miR-138	P53 Pathway	Promoters	Ye *et al*., 2012

(DNMTs) act as barriers to early stage reprogramming. However, its expression is up-regulated during later stages of iPSCs generation (Pawlak and Jaenisch, 2011). Guo *et al*. (2012) demonstrated that Sox2 directly regulates miR-29b expression during reprogramming and expression of miR-29b is essential for OSKM- and OSK-mediated reprogramming. miR-29b targets Dnmt3a and Dnmt3b, thus enhancing expression of MET promoting factors, Cldn3, E-Cadherin and EPCAM, while suppressing expression of mesenchymal specific genes like Cdh2, Snail and Zeb1 during reprogramming events (Guo *et al*., 2012). Expression of miR-29b in OSKM-mediated iPSCs generation with low transcriptional activity of the Dlk1-Dio3 locus reactivates expression of miRNAs and genes in the imprinted region (Stadtfeld *et al*., 2012) (Fig. 2).

miR-302b (orthologous to mouse miR-302s) and miR-372, miR-373 (orthologous to mouse miR-291, miR-294, miR-295) enhances human somatic cell reprogramming by increasing the kinetics of MET, by suppressing TGF-β induced EMT and by targeting epigenetic modifiers (MECP2, MBD2, SMARCC2) (Subramanyam *et al*., 2011). In addition, miR-302 expression in reprogramming leads to DNA hypomethylation and DNMT1 deficiency (Lin *et al*., 2011). In conclusion, these findings

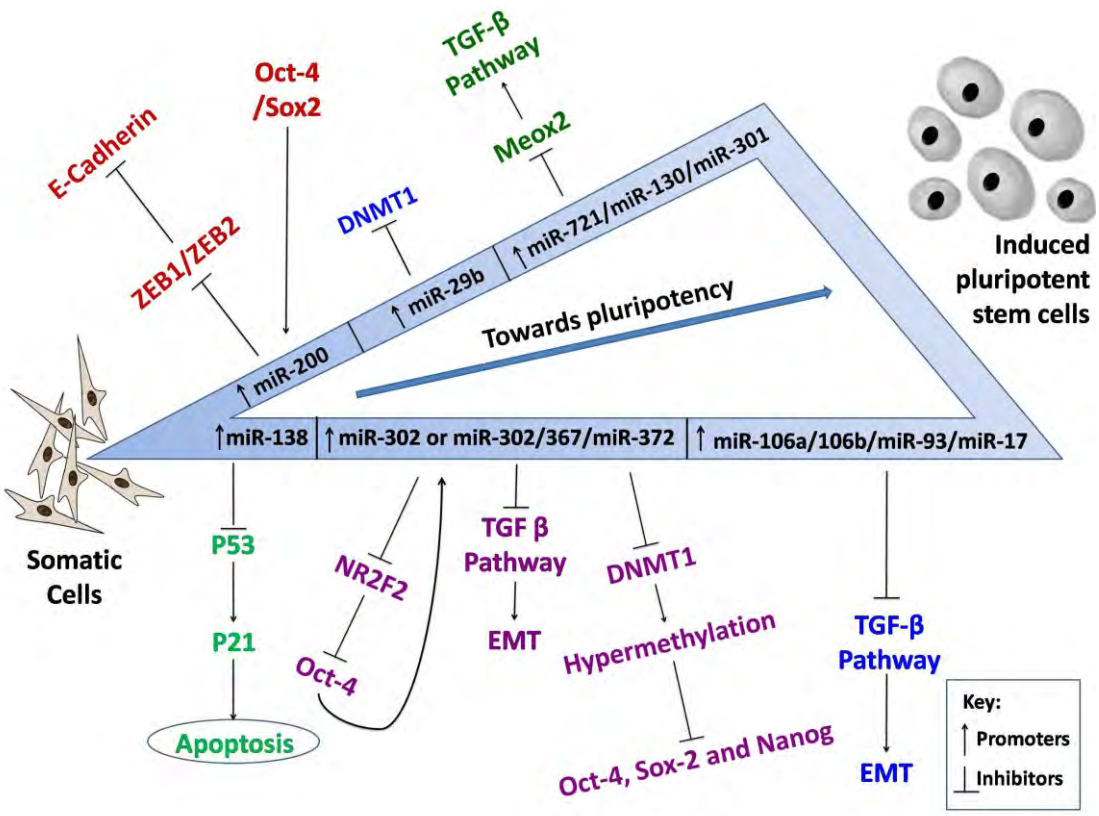

Figure 2: miRNAs that modulate reprogramming along with their downstream effectors. miR-138 promotes reprogramming by suppressing inhibiory effects of P53 pathways. P53 induces apoptosis by promoting expression of cyclin-dependent kinase P21. miR-302 cluster or miR-302/367/372 has multiple targets. miR-302 suppresses expression of Oct-4 inhibitor, NR2F2, cluster inhibits TGF β pathway thus blocking EMT transition and augments expression of pluripotent genes, Oct-4, Sox-2 and Nanog by inhibiting DNMT1 thus suppressing hypermethylation. miR-106a, miR-106b miR93 and miR17 accelerate reprogramming by targeting MET inhibitor TGF βII. Pluripotent genes, Oct-4/Sox-2 positively regulate expression of miR-200 which in turn down regulates transcriptional repressor of E-Cadherin, ZEB1/ZEB2 expression. miR-29b inhibits DNMT1. miR-130, miR-301 and miR-721 cluster of miRNAs enhance mouse fibroblast reprogramming by reducing expression of homeobox transcription factor, Meox-2. Abbreviations – miRNAs: microRNAs; NR2F2: Nuclear receptor subfamily 2, group F, member 2; TGFβ: Transforming growth factor beta; DNMT: DNA methyltransferase; MET: Mesenchymal-to-epithelial transition; EMT: Epithelial-to-mesenchymal transition; ZEB: Zinc finger E-box binding homeobox.

show that miRNAs are necessary for gene expression and epigenetic remodeling during OSKM-mediated somatic cell reprogramming. Thus, it is observed that miRNAs are differentially expressed and possess critical functions during reprogramming of somatic cells.

ESCC regulating miRNAs for reprogramming

The embryonic stem cell-specific cell cycle-regulating (ESCC) family of miRNAs promote reprogramming of somatic cells to iPSCs (Judson *et al*., 2009; Subramanyam *et al*., 2011). The role of ESC-specific miRNAs in iPSCs generation was first demonstrated by

Judson and Blelloch (2009). MEFs were reprogrammed by viral transfection vector system using transcription factors Oct4, Sox2 and Klf4 (OSK) and miRNA-290 cluster mimics including miR-291 – 3p, miR-292-3p, miR-293, miR-294, and miR-295. The reprogramming efficiency enhanced with miR-291-3p, miR-294, and miR-295, whereas miR-292 and miR-293 were not effective. Optimum results were obtained by overexpressing miR-294, with increasing efficiency from 0.01–0.05% to 0.1–0.3%. These reports show that miR-294 can substitute for c-Myc in order to enhance reprogramming in presence of other transcription factors (OSK) (Judson *et al*., 2009). Importantly, iPSCs generated without c-Myc will be safer for future use in clinical research. miR-290 (mouse), miR-372 and miR-302 cluster, ESCC specific miRNAs directly target inhibitors of cyclin-Cdk2 pathway, thereby ensuring fast G1-S transition. The miRNAs has reported to augment reprogramming of human fibroblasts (Guo *et al*., 2014; Subramanyam *et al*., 2011) (Fig. 2 and Table 1). Bone morphogenic protein (BMP) is necessary for efficient reprogramming along with OSKM, promoting MET by inducing expression of miR-200 and miR-205 (Tehrani *et al.,* 2010). The various targets of miR302/367 were revealed using photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation method (PAR-CLIP). miR-302/367 promotes BMP signaling by targeting BMP inhibitors TOB2, DAZAP2 and SLAINI (Lipchina *et al.,* 2011)

miRNAs modulate late stages of reprogramming

Activation of pluripotent markers occurs in late stages of reprogramming. miR-302/367 regulates expression of pluripotent markers, Oct-4, Sox-2 and Nanog (Hu *et al*., 2013; Marson *et al*., 2008; Rosa and Brivanlou, 2013). Human adipose tissue derived stem cells (hASCs) were reprogrammed into iPSCs using Yamanaka factors (Klf4, c-Myc, Oct4, and Sox2) with miR-302 (combination is referred to as 'KMOS3'). Thus, miR-302 blocks expression of Oct-4 inhibitor, NR2F2 and promotes pluripotency by regulating Oct-4 through indirect mechanism. The positive feedback loop represents a novel mechanism for inducing pluripotency status in somatic cells (Hu *et al*., 2013). Study in hESCs, showed that expression of NR2F2 increases with differentiation and simultaneously down regulation of Oct-4 and miR-302/367 expression was observed (Marson *et al*., 2008). The transcription factors, Oct-4, Nanog and Sox-2 enhances expression of miR-302/367 by binding to its promoter region (Anokye-Danso *et al*., 2012; Marson *et al*., 2008). miR-302/367 cluster indirectly modulates expression of multiple pluripotent factors by targeting several epigenetic modifiers leading to global genomic

hypomethylation. Often, genomic hypomethylation occurs at the promoter region of ESCs specific important transcription factors (Lin *et al.,* 2011). During somatic reprogramming, miR-302 supresses expression of DNA methyltransferases 1 (DNMT1) which inhibits expression of AOF2 (lysine specific histone DNA methylases). This leads to genomic hypomethylation and subsequently reactivation of essential pluripotent factors (Lin *et al*., 2011; Reik *et al*., 2001) (Figs. 1 and 2). Tay *et al.* (2008) demonstrated that miR-134, miR-296 and miR-470 act as barriers to reprogrammimg by inhibiting pluripotent transcription factors.

miRNAs alone for reprogramming

The strategies currently employed for iPSC generation involves, ectopic expression of Yamanaka factors (OSKM) (Takahashi and Yamanaka 2006; Takahashi *et al*., 2007). Although numerous alternate approaches have been documented to augment iPSC generation, including use of signaling molecules, additional transcription factors and pharmacological molecules (Jia *et al*., 2010), the different approaches require at least one pluripotent stem cell transcription factor. Lin *et al.* (2011) have reported use of miRNA-302 cluster for successful reprogramming without need of any transcription factor. There are four major advantages in miRNA-based reprogramming, compared to conventional methodology used for reprogramming. Firstly, the transfection of miRNA cluster expressing transgene is safe, easy and efficient for generating iPSCs, thus bypassing the tedious adeno- or retro-viral insertion of huge transcription factors (OSKM) into single somatic cell. Secondly, since the size of miRNA transgene is approximately 1kb the efficiency of transfection will be increased. Thirdly, generation of iPSCs by using miRNA-based approach circumvents use of proto-oncogenes. Several investigators have employed exclusively miR-302a/b/c/d or in combination with miR-302a/b/c/d and miR-369 or miR-302a/b/c/d and miR200c and miR-369 for reprogramming without oncogenes, c-Myc or Klf-4 (Anokye-Danso *et al*., 2011; Lin *et al*., 2011; Miyoshi *et al*., 2011). Finally, the transfection of miRNA cluster transgene is done by electroporation instead of retroviral vector system (Lin *et al*., 2011). Hence, somatic cells can be successfully reprogrammed without use of pluripotent transcription factors in the miRNA-based approach (Anokye-Danso *et al*., 2011; Lin *et al*., 2011). miRNA-based reprogramming approach has circumvented most of the problems encountered in SCR using conventional method. The future challenge will be to apply this technique to generate patient-specific iPSCs in a large scale with better quality and safety for transplantation therapy.

miRNAs in regulation of lineage differentiation

A self renewal process is normally inhibited during differentiation due to down regulation of pluripotent genes, Oct-4, Sox-2 and Nanog, hence resulting in a decrease of miR-290 and lin-28 cluster expression. A down regulation of lin-28 leads to maturation of let-7, resulting in the suppression of self-renewal promoting genes, hence facilitating differentiation. miR-290 family indirectly represses let-7 in order to maintain pluripotent state (Guo *et al*., 2014). Depletion of miR-290 family results in differentiation of pluripotent stem cells. Bernardini *et al*. (2014) reported crucial role of miR-21 during endodermal differentiation of iPSCs. PTEN/Akt pathway is a direct target of miR-21 and augments TGF-β2 expression, thus promoting endodermal differentiation of iPSCs. Okamoto *et al.* (2012) demonstrated that miR-181a, miR-24a, miR-9-3p, miR-19b, miR-10b, miR-10a are important regulatory factors in osteoblastic differentiation of mouse iPSCs. Specifically, miR-124a and miR-181a directly targets the transcription factors Dlx5 and Msx2. Hence, down regulation of these miRNAs are necessary to enhance expression of osteoblastic differentiation markers such as Rux2, Msx2 and osteopontin.

SUMMARY

Somatic cell reprogramming is a ground breaking discovery in the field of stem cells and regenerative medicine. The iPSC technology has opened avenues for personalized medicine, since patient-specific somatic cells can be employed. However, to realize the therapeutic potential of iPSCs, comprehension of the molecular mechanisms involved in pluripotency and cell fate decisions are critical. Despite numerous advancements in iPSC research, the search for a methodology that is safe, effective with high efficiency, for engineering somatic cells into a versatile embryonic-like state is ongoing. miRNA-based reprogramming methods seem promising for generation of iPSCs/progenitor cells using defined approaches, and will be more efficient than the conventional (Oct 4/Sox2/Klf4/c-Myc mediated) methods. miRNAs are differentially expressed in an organized manner during the entire reprogramming process. A key cellular process, MET, is an early stage event that occurs immediately after forced expression of core transcription factors required for reprogramming in fibroblasts. Simultaneously, inhibition of EMT is a prerequisite for efficient reprogramming. A distinct set of miRNAs modulate the EMT/MET transitions, a critical step towards an altered pluripotent state. Finally, another distinct set of ESC specific miRNAs is observed, ensuring that the pluripotency regulatory network is maintained. miRNA-based reprogramming methods are relatively new and a number of challenges have to be addressed including, the ideal cluster of

miRNAs for reprogramming, the different types of clusters that can be used for reprogramming at a single time point, a distinct signature of miRNAs that could serve as a fingerprint for a particular stage of reprogramming. We have progressed immensely in understanding of several molecules for reprogramming with several unknowns to be unraveled.

CONFLICT OF INTEREST

The authors claim no conflict of interest.

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Cultivation and Cryopreservation of Cord Tissue MSCs with Cord Blood AB Plasma

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Neonatal tissues, cord and placenta, are explored as alternate sources of mesenchymal stem cells (MSCs) for their therapeutic applications. Conventionally, MSCs isolated from cord tissues are maintained and propagated in FBS containing medium for promotion of growth and survival of cells. However, for therapeutic use, FBS use is not encouraged as it is of animal origin. Thus, there is a need for replacement of FBS by equally potent and clinically acceptable cost effective sources. The current study is designed to compare the effect of cord blood plasma (CBP) with MSC qualified FBS (M FBS) during culture and cryopreservation of MSCs. MSCs were isolated from cord and placenta and propagated in either M FBS or CBP. The efficiency of the cultures was analyzed by growth curve, morphology, phenotype and functionality. The cryo-protective role of the CBP was evaluated by using it in freezing medium of MSCs. Our data showed that CBP is equivalent to M FBS for culturing placental MSCs with respect to the phenotype, proliferation rate and differentiation to various lineages. However, cord MSCs displayed slow growth rate and reduction in surface expression of CD105 marker in CBP, whereas, the other parameters were comparable. Freezing of MSCs with CBP resulted in reduction of the late apoptotic and necrotic population. Thus, CBP imparts superior protection against cryogenic insults, and appears to be a valuable substitute to M FBS for cultivation and freezing of MSCs.

INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells with multi-differentiation potential. MSCs have gained a substantial therapeutic value because of their plasticity and immune suppressive characteristics (Knaan-Shanzer, 2014). MSCs are primarily isolated from bone marrow (BM) aspirates. Owing to the invasive procedure and limitation in amount of sample procured, alternate sources such as umbilical cord, placenta, endometrial polyps, menstrual blood, adipose tissue, are being harnessed to obtain MSCs (Moroni and Fornasari, 2013).

Cord/placenta derived MSCs need to be cryopreserved so that they can be revived and used in clinics when required. Generally, the protocols use specialized FBS like mesen FBS (M FBS) for culturing and freezing MSCs (Ng *et al*., 2008; 2014). However, clinical transplantation protocols demand replacement of xenogeneic products to avoid adverse

Key words: Mesenchymal stem cells, cord, placenta, mesen FBS, cord blood plasma.
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immunological reactions and possible transmission of infectious agents (Ma *et* al., 2012). Several reports indicate use of serum free media for culture and expansion of MSCs (Al-Saqi *et al*., 2014). However, the protocols are expensive and not as effective as serum containing media. Thus, there is a need for substitutes for xenogeneic sera including autologous serum (Wang *et al*., 2012), autologous plasma (Lin *et al*., 2005), PRP (Pham *et al*., 2014), platelet lysates (Iudicone *et al*., 2014). However, availability of human blood, plasma, platelets from blood banks is difficult, expensive and considered unethical. Cord blood banking opened a new avenue for regenerative medicine. During banking of the samples, the RBCs are separated to obtain the mononuclear cells (MNCs) and hematopoietic stem cells (HSCs). At this time if the plasma is collected and stored separately, it can be utilized for culturing cord/placenta MSCs and thus the fractionated products of cord blood can be put to use in the clinics**.**

In the current article, cord blood derived AB positive plasma (CBP) has been used for both culture and freezing of MSCs isolated from cord (C MSCs) and placenta (P MSCs) and its efficiency compared with M FBS. Our data indicates that CBP can serve as a valuable substitute to FBS, and be acceptable in the clinics.

MATERIALS AND METHODS

Isolation of MSCs

Umbilical cord and placenta were collected from full term deliveries from clinics and from independent donors. The collection was performed with informed consent as per the protocols approved by the Institutional Ethics Committee (IEC), NCCS, Pune (11th IEC/IC-SCRT of NCCS held on 20th Jan 2012). Umbilical cord and a section from the central part of the placenta were used to isolate the MSCs. The tissues were washed in Iscove's modified Dulbecco's medium (IMDM) (Sigma Aldrich, St Louis, USA) and chopped into pieces and then subjected to enzymatic digestion with 0.25% trypsin for 30 min. Single cell suspension was obtained by passing the homogenate through sterile muslin cloth. The cells were washed with medium and suspended in complete growth medium RPMI 1640 (Sigma Aldrich, St Louis, USA) with 20% M FBS (Gibco, Invitrogen, Grand Island, USA) and seeded in 75 cm^{2}tissue culture flasks (Falcon, Beckton Dickinson, San Jose, CA, USA). Non-adherent cells were removed after 72 h. Cultures were re-fed with fresh medium after every 72 h for 10–20 days. The cultures were maintained by passaging them after reaching 70–80% confluency, for 6–7 passages. The cells between passage numbers 3–6 were used for the experiments after confirming the phenotypic signature of the MSCs. Adaptation of cultures to CBP was initiated in passage 2, described below.

Collection of AB positive plasma from cord blood

Cord blood samples were collected from local hospitals with the compliance of the institutional review board (IEC, NCCS).

Blood grouping was carried out with ERYSCREEN reagent (Tulip Diagnostic, Mumbai, India). From the AB positive samples, mononuclear cells and plasma were simultaneously isolated by a single step method using Ficoll Hypaque (Sigma Aldrich, St. Louis, MO, USA) density gradient separation (Density 1.077 g/ml). The separated plasma was collected and complement inactivation was carried out at 56 ºC for 30 min. After removing aliquots for sterility checking, the plasma was stored frozen at –20 ºC and further used for maintenance of MSCs. As AB plasma is only used for the experimental purpose the levels of endotoxin were not checked. The MNCs obtained during plasma collection were utilized for the isolation of hematopoietic stem cells. AB positive plasma from three independent cord blood units were used for the experiments.

Culture adaptation and propagation of MSCs to CBP

For adaptation to CBP we used MSCs from passage 2 (60–70% confluent), washed 3–4 times with PBS before changing the plasma containing medium. C MSCs (n = 3) and P MSCs (n = 3) in passage 3 were harvested and divided in two sets, and cultured in RPMI + 20% M FBS and RPMI + 20% CBP, with equal seeding density in T25 cm^2 flasks. After the cells reached confluence they were harvested and used for subsequent experiments. Traces of FBS after CBP adaptation were not checked.

Growth kinetic study

1×10^3 cells were seeded in 96-well plates in RPMI + 20% M FBS/CBP containing medium. Proliferation was assessed by MTT assay (Sigma Aldrich, St Louis, USA). The cultures were followed for 144 h and absorbance measured at 570 nm.

Characterization of MSCs by flow cytometry

MSCs expanded from cord or placental tissue were characterized by the following antibodies – CD105-PE, CD73-APC, CD166-PE, CD90-APC, CD34-PE (Beckton Dickinson Pharmingen, San Jose, California, USA) and CD45-APC (eBiosciences, San Diego, USA). Isotype matched antibodies were kept as controls. The fluorescent labeled cells were acquired on fluorescence activated cell sorter (FACS) Canto II (Beckton Dickinson, San Jose, CA USA). An acquisition of 10,000 events was done and data analyzed by FACS DIVA, version 5.0.

Colony forming unit – Fibroblast assay (CFU-F)

5×10^3 MSCs were seeded in 60 mm dish with RPMI + 20% M FBS/CBP containing medium and incubated at 37 °C, 5% CO_2 humidified environment for 7–10 days. The non-adherent cells were removed, the monolayer was washed with PBS and fixed using 100% methanol (Fischer Scientific, Mumbai, India) for 10 min. The cells were stained with 0.1% crystal violet solution for 10 min. Clones of > 50 cells were scored as CFU-F. Staining was done in triplicates for each sample.

Differentiation to osteoblasts and adipocytes

1×10^4 MSCs were seeded in 24-well plates (Falcon, Becton Dickinson, San Jose, USA) and grown to 60–70% confluency. The cells were then subjected to osteogenic and adipogenic differentiation for 15–18 days using kit (STEMPRO® Osteogenesis/ Adipogenesis Differentiation Kit, Invitrogen, USA). The osteogenic and adipogenic differentiation was confirmed by performing Alizarin Red S (Sigma Aldrich, St Louis, USA) staining for calcium deposits and intracellular lipid droplets staining with Oil red O dye (Sigma Aldrich, St Louis, USA), respectively.

Cryopreservation of MSCs

1×10^6 MSCs were cryopreserved in conventional freezing medium (RPMI + 20% M FBS/cord blood plasma**)** containing 10% DMSO by portable programmable freezer (Freeze Control, Victoria, Australia) at the controlled cooling rate of 1 ºC/min to –40 ºC followed by 10 ºC/min to –90 ºC and then kept in liquid nitrogen. The cells were thawed by rapidly immersing the vials in a water bath at 37 ºC. The viability of the cells was assessed by trypan blue dye exclusion method. Further, apoptotic profiling was carried out by performing Annexin V and PI staining (Becton Dickinson Biosciences, San Jose, CA, USA) as per manufacture's instructions and analyzed by flow cytometry.

Statistical analysis

The differences between growth of C MSCs in FBS and C MSCs in CBP (n = 3), and between growth of PMSCs in FBS and P MSCs in CBP (n = 3) were compared by a one way repeated measure analysis of variance using the software SIGMA STAT (Jandel Scientific Corporation, San Rafael, CA, USA). The values were plotted as mean ± standard deviation. Probability values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Morphological differences in MSCs cultured in M FBS vs CBP

MSCs cultured in M FBS and CBP showed typical fibroblast morphology (Fig. 1A–D). However, C MSC and P MSC growth in CBP was aberrant, with persistent clusters observed in the cultures. The clusters were more prominent in C MSCs (Fig. 1B) as compared to P MSCs (Fig. 1D) indicating a delayed growth of C MSCs as compared to P MSCs. This pattern of growth in C MSCs may be attributed to low seeding density as this trend was reversed by increasing seeding densities.

C MSCs grown in FBS show higher proliferation rate

Growth kinetics of the MSCs in M FBS vs CBP were investigated to check whether the differences in morphological appearances of the cultures were reflected in the proliferation rates. The C MSCs with M FBS showed higher growth rates as compared to those grown in

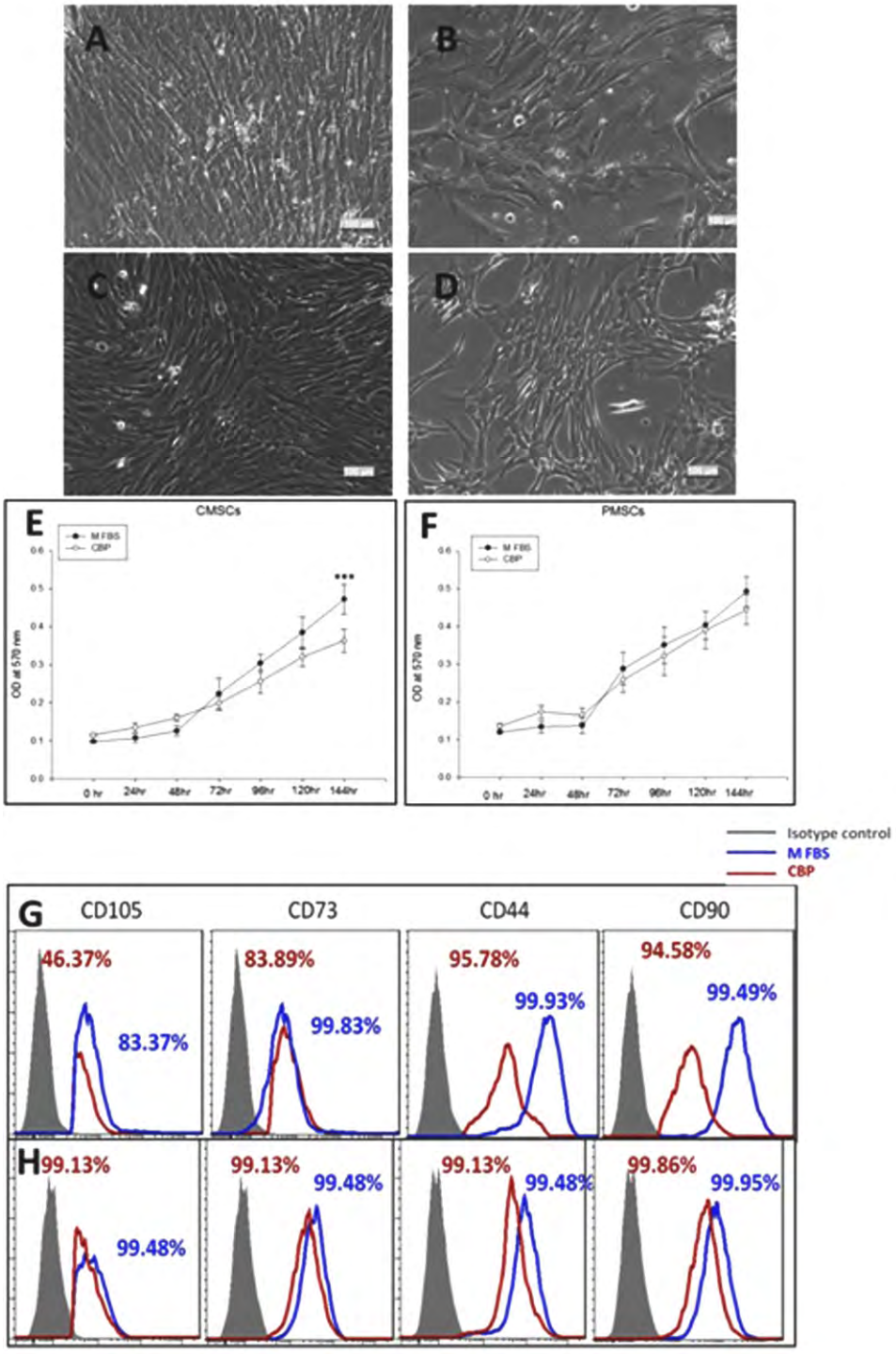

Figure 1: Morphology, growth kinetics and phenotype of MSCs grown with M FBS and CBP: Phase contrast images of C MSCs and P MSCs grown in M FBS (A and C, respectively) and CBP (B and D, respectively). Comparison of growth kinetics of C MSCs (E) and P MSCs (F) in M FBS and CBP. Surface marker analysis by flow cytometry of MSCs grown in either M FBS or CBP in both C MSCs (G) and P MSCs (H).

CBP and the difference was significant at day six (Fig. 1E). However, P MSCs showed more or less similar proliferation patterns with FBS/plasma (Fig. 1F).

P MSCs grown in M FBS/CBP show equivalent level of expression of surface markers

We next proceeded to characterize the MSCs grown with FBS/plasma for the expression of surface markers. Significant differences were not observed with P MSCs/C MSCs in both media for surface marker expression of CD73, CD90 and CD44. However, C MSCs showed 50% less expression of CD105 when grown in AB plasma. Histogram overlays for a representative sample for using a panel of antibodies are depicted in Figs. 1G and H for C MSCs and P MSCs, respectively. The MFI levels were increased in C MSCs with M FBS as compared to those seen in CBP.

MSCs cultured in CBP have comparable CFU-F potential and differentiation ability

The clonogenicity of MSCs was assessed by CFU-F assay. MSCs in CBP showed 120 ± 5 CFU-F and in M FBS showed 150 ± 10 CFU-F per 5×10^3 cells indicating comparable potential in both media. Differentiation of MSCs to adipocytes and osteoblasts was similar in M FBS and CBP containing media. Images of CFU-F (Fig. 2A) and differentiation to adipocytes (Fig. 2B) and osteoblasts (Fig. 2C) of representative MSCs in M FBS and CBP are depicted.

Cord plasma is a useful substitute to FBS for cryopreservation of MSCs

The cells are subjected to stress during cryopreservation and may be enhanced if the culture medium and freezing media are different. Hence, C and P MSCs grown with M FBS and CBP were cryopreserved in the respective media containing 10% DMSO and 20% FBS/Plasma. The viability post revival by trypan blue dye exclusion test ranged from 75–80% in all the sets. Cryopreserved MSCs were revived and assessed for retention of the cell surface markers. Post thaw phenotypic analyses did not reveal any significant difference in expression of markers like CD44, CD73, and CD90 in the two sets, except with the CD105 expression in C MSCs (Table 1).

The revived MSCs from the two sources frozen with two different media were subjected to Annexin V–PI staining and analyzed on flow cytometry for the percentage of viable, apoptotic and necrotic cells. C MSCs frozen with FBS showed less (48.00 ± 6.23%) viable cells as compared to CBP (61.10 ± 9.93%), whereas P MSCs showed similar number of viable cells (58.57 ± 10.93%) in the two sets. Lesser percentage (9.27 ± 3.51% and 3.10 ± 1.37%) of early apoptotic cells and more (24.87 ± 9.05% and 23.65 ± 7.27%) late apoptotic and necrotic cells (17.8 ± 6.38% and 14.72 ± 2.28%) were observed in both C and P MSCs frozen in M FBS, respectively. On contrary, the percentage of late apoptotic and necrotic cells were significantly reduced (less than 10%) for both

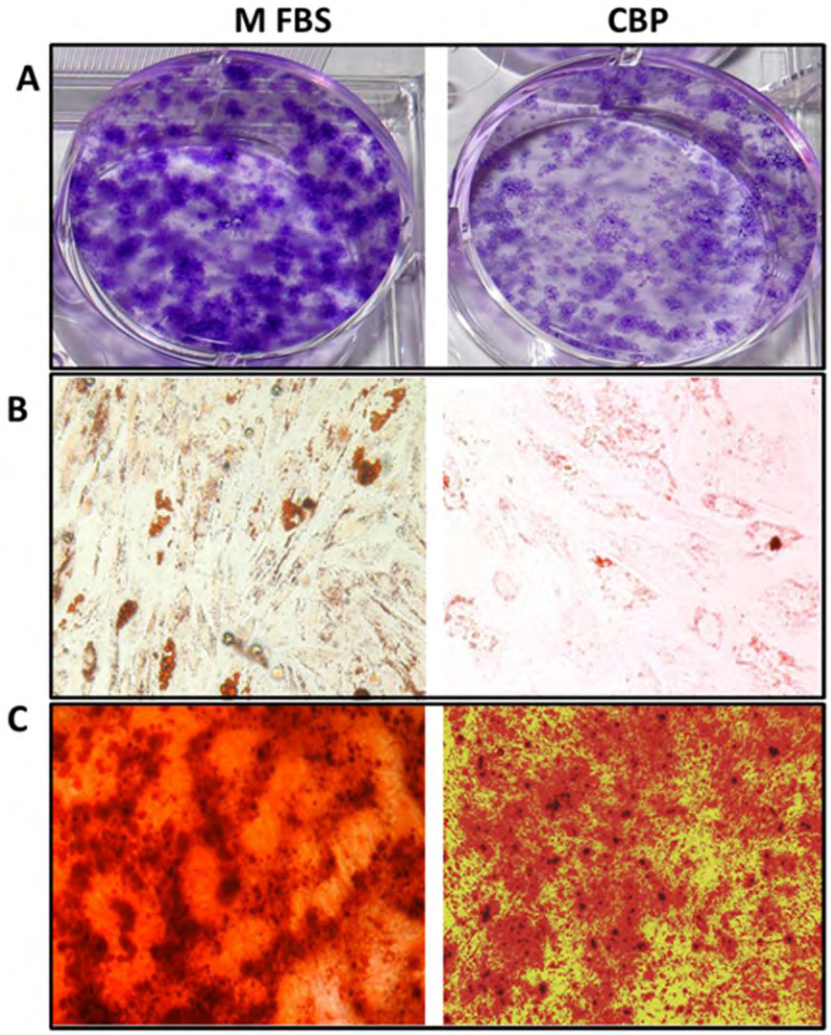

Figure 2: Microphotograph depicting *in vitro* functionality of MSCs and CBP: (A) Distribution of colonies of MSCs in CFU-F cultures grown in M FBS and CBP. MSCs cultured in M FBS and CBP subjected to (B) adipogenic and (C) osteogenic differentiation and stained with oil red O and Alizarin red S, respectively.

C and P MSCs frozen with CBP with a relatively higher percentage of early apoptotic population (36.33 ± 9.80% and 32.60 ± 10.89%) (Fig 3A). A representative FACS profile is depicted in Fig 3B. This data suggests that CBP is more efficient than M FBS in protecting the MSCs from cryo injuries.

DISCUSSION

BM-MSCs are difficult to obtain and their quality deteriorates with age. So cord tissue derived MSCs are an alternative for cell based therapies (Moroni and Fornasari, 2013). Ready availability and ontogenetically conserved nature of the umbilical cord tissues prompted us to assess cord and placenta for

Table 1: Post thaw phenotpic analysis for expression of markers in C MSCs and P MSCs (**P< 0.01).

	C MSCs		P MSCs	
Marker	**M FBS**	**CBP**	**M FBS**	**CBP**
CD105	83.03 ± 12.77	35.06 ± 7.52**	75.5 ± 24.09	76.06 ± 23.89
CD44	97.23 ± 1.49	99.8 ± 0.1	97.93 ± 0.49	99.93 ± 0.05
CD73	97.8 ± 1.24	95.86 ± 1.02	98.33 ± 0.58	97.83 ± 2.54
CD90	97.63 ± 1	99.7 ± 0.36	95.87 ± 2.45	99.46 ± 0.75
CD34	0.43 ± 1.02	0.4 ± 0.26	0.57 ± 1.46	0.76 ± 0.25
CD45	0.67 ± 1.06	2.70 ± 0.69	–0.43 ± 0.4	3.2 ± 0.3

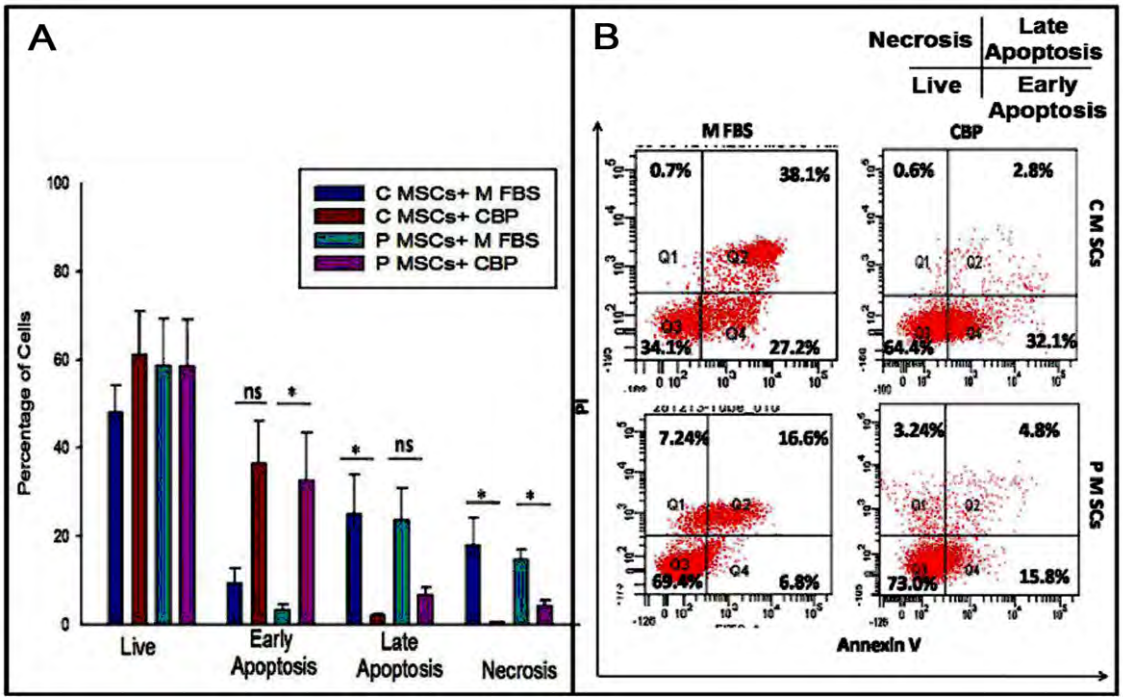

Figure 3: Revival of MSCs froen in M FBS/CBP: (A) Annexin V staining shows significant decrease in the late apoptotic and increase in the early apoptotic population when MSCs were frozen with CBP; (B) Representative FACS profiles of Annexin V and PI staining for MSCs frozen/revived with M FBS and CBP, respectively . *P< 0.05.

isolation of MSCs. No significant difference was observed in major characteristics including morphology and phenotype of MSCs obtained from the two sources (data not shown). However, inherent differences in these populations need to be extensively investigated.

MSCs are generally propagated in serum containing media. Due to the xenogeneic nature of FBS, an elevated risk of transmitting infectious agents and adverse immunological reactions is a distinct possibility (Ng *et al*., 2008; 2014). In the current study we have attempted to culture and cryopreserve

clinically compliant MSCs by replacing M FBS with CBP in the media. We initially isolated the MSCs in the FBS containing medium as FBS has cell stimulatory and survival promoting factors essential for the initial survival. The MSC colonies were adapted to CBP. The aim of the study was to culture and adapt the MSCs to AB positive CBP. Due to the availability of cord blood, as often it is discarded, CBP is a cost effective substitute for FBS. We report the use of CBP for cultivation and cryopreservation of MSCs isolated from both cord and placenta. We preferred plasma over serum to curtail loss of precious stem cells within the clotted fraction while collecting serum. Autologous plasma from human source has been studied in the context of BM-MSCs. As we are studying cord/placenta derived MSCs, CBP was preferred to peripheral blood derived plasma as a suitable substitute for FBS which contributes to xenogeneic proteins. Considering the fact that under *in vivo* conditions placenta, cord and cord blood demonstrated similar interactions, the cultures may adapt to CBP. AB positive plasma was the choice due to absence of antibodies for either antigen which may influence the growth and development of MSCs.

Our data demonstrated CBP as an effective substitute to M FBS as characterized by the growth pattern, phenotypic signature, clonogenic ability and differentiation capabilities of both C MSCs and P MSCs. However, the observed lag in the growth kinetics for C MSCs in CBP may be attributed to the delay in the adaptation to the serum replacement. The lag period can be shortened by increasing the seeding densities during the initial passages, whereas P MSCs showed comparable growth in both media. The immunophenotype of the both MSCs was similar with the exception of reduced CD105 expression by C MSCs in CBP. Mark *et al*. (2013) have reported decreased CD105 expression in serum free medium as compared to serum containing media. Our data showed comparable CD105 expression for PMSCs which may be attributed to a more adaptable nature of the cells. A functional assay for MSCs is the multi-lineage differentiation capacity of the cells. Our data showed that MSC adaptation to CBP retained the multi-lineage differentiation capacity with minor variations in the differentiation exhibited by MSCs grown in FBS vs CBP. It has been reported that variations in culture conditions influence the differentiation potential of MSCs (Al-Saqi *et al*., 2014). Perhaps the observed differences may be attributed to a proliferation delay in CBP containing cultures. Cryopreservation of MSCs in chemically defined media is essential for application as ready to use off the shelf cell products in therapeutics. Roy *et al*. (2014) replaced serum by sucrose during cryopreservation of MSCs and reported that the cells were compromised. We have demonstrated CBP as a better cryoprotectant over conventional FBS in the freezing medium. Substitution of FBS with CBP in the freezing medium in our studies had no adverse effect on viability of the cells as

detected by both trypan blue dye exclusion and Annexin V-PI staining. Interestingly, MSCs frozen with CBP exhibited enhanced protection from cryo-injury, as there was a significant reduction in the late apoptotic and necrotic population as opposed to MSCs frozen with FBS. Though we observed an increase in the early apoptotic population (Annexin V^+ PI^-) for CBP set as compared to FBS set, this population can be rescued back to the Annexin V^- PI^- double negative phenotype (viable) by continual maintenance at 37 ºC (Chinnadurai *et al*., 2014; Geske *et al*., 2001).

Recently, Ng *et al*. (2014) described fetal extracellular matrix proteins for culturing adult MSCs, thus providing natural biological supports compared to synthetic polymers. With an increasing trend towards use of natural non-xenogenic substitutes for culture, cryopreservation and expansion of MSCs, CBP is a potential, economical and valuable substitute for FBS in culture and freezing media for MSCs. However, adaptation of the cultures for a few passages to improve growth patterns and expression of surface molecules while retaining their genetic stability needs to be explored. Further, prior to use of CBP adapted MSCs presence of endotoxins should to be checked.

Our data suggests that CBP may be a relevant substitute for FBS in clinical applications.

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CONFLICT OF INTEREST

The authors claim no conflict of interest.

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Implications of Cancer Stem Cells in Radiotherapy Current Understanding and Future Perspectives

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Many theories were put forward about origin of cancer and immense research was carried out to understand the mechanisms involved in the origin and progression of the disease. The gain in scientific knowledge about cancer biology and technical advancement in treatment modalities resulted in improvement of clinical outcome in cancer therapy. However, recurrence and metastasis after therapy poses a major concern to clinicians. Resistant nature against therapeutic modalities and metastatic potential of cancer stem cells (CSCs), which even though form a fraction of tumor mass, result in failure of existing modalities of cancer treatment. The current article reviews the historical background of CSCs, involvement of various signaling pathways in the mechanism(s) of radioresistance and potential targets to be exploited in radiotherapy.

INTRODUCTION

Cancer stem cell research has gained substantial attention by biomedical scientists and clinicians in the recent years due to growing realization of their role in various aspects of cancer biology (progression, metastasis) and therapy (chemo- and radio-resistance). The concept of 'cancer stem cell' (CSC) was proposed in 1863 by Rudolf Virchow, an eminent pathologist, who observed the abnormal mixture of undifferentiated embryonal cell with differentiated adult cells in teratocarcinomas. Later on, Cohnheim, a student of Rudolf Virchow, put forward 'embryonic rest theory' in 1875, which states that during embryonic development, certain cells became isolated and manifested their uncontrolled proliferative potential during adult life (Cohnheim 1875; Sell, 2004; Virchow 1863). Similarities in growth characteristics (embryoid bodies) and histopathological features (undifferentiated cells) exist in embryonic tissues and teratocarcinomas. These features of teratocarcinomas led to postulate that the tumors might have originated from undifferentiated stem-like cells (Bignold *et al*., 2006; Pierce *et al*., 1959; 1960). Later in 1930s, Furth *et al*. (1937) demonstrated that a single malignant white blood cell is capable of producing leukemia, a systemic disease in mice. These noteworthy

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developments in cancer stem cell biology at that stage were not pursued extensively and were overshadowed due to gaining significance of mutation associated with carcinogens in tumor development (Bishop 1985; Rous 1983; Weinberg 1985; Yamagiwa *et al*., 1977). The area of cancer stem cell biology remained relatively dormant till late twentieth century until Lapidot *et al*. (1994) demonstrated that a small subset of cancer cells ($CD34^{+}CD38^{-}$) sorted from the blood of acute myeloid leukemia patient resulted in development of leukemia in SCID mice (Lapidot *et al*., 1994), whereas the $CD34^{+}CD38^{+}$ cells did not possess the ability to develop leukemia (Bonnet and Dick 1997; Lapidot *et al*., 1994). In 2003, CSCs were first identified in solid tumors by Al-Hajj and colleagues in breast cancer patients (Al-Hajj *et al*., 2003), where 100 $CD44^{+}/CD24^{-/low}$ cells injected underneath the mammary pad of non-obese diabetic (NOD) mouse strain, an excellent model of autoimmune disease, or SCID mice resulted in tumor formation. However, cells with $CD44^{+}CD24^{+}$ phenotype did not form tumors even when injected in thousands. Eventually, other solid tumors like brain, prostrate, lung, gastric, head and neck tumors also demonstrated presence of CSCs (Albers *et al*., 2012; Collins *et al*., 2005; Eramo *et al*., 2008; Singh *et al*., 2003; Takaishi *et al*., 2009; Tirino *et al*., 2013). Recently, it was also shown by lineage tracing experiments in mouse models that CSCs are responsible for tumor formation and are resistant to chemotherapy (Driessens *et al*., 2012; Schepers *et al*., 2012). Once treated by chemotherapy, these cells survive the therapy and responsible for the re-growth of tumor (Chen *et al*., 2012). It was hypothesized that the tumor stem cell divides by asymmetric division resulting in the formation of CSC and differentiated cell. Further, these cells divide and form heterogeneous tumor mass comprising various tumor tissue cells and these cells vary according to tumor type and alter during the course of cancer therapy (Fig. 1). The interactions between these various tumor cells and CSCs are hypothesized to play critical role in mechanism of radioresistance and unraveling these mechanisms may have significant implication in clinical outcome of cancer radiotherapy (Peitzsch *et al*., 2013).

CSCs in Mechanism of Radioresistance

Radioresistance is a major challenge in therapeutic outcome of cancer, associated with magnitude of CSCs in tumour mass (Chen *et al*., 2013; Shiozawa *et al*., 2013). Hence, the cure of cancer may depend upon targeting the resistant CSCs. The idea is supported by studies suggesting lower radiation induced apoptosis in glioblastoma cells expressing CD133 (Bao *et al*., 2006). In addition, the cells expressing CD133 increased significantly after radiation treatment in xenografts of glioblastoma (Bao *et al*., 2006). In the breast carcinoma cell line, MCF7, a sub-population

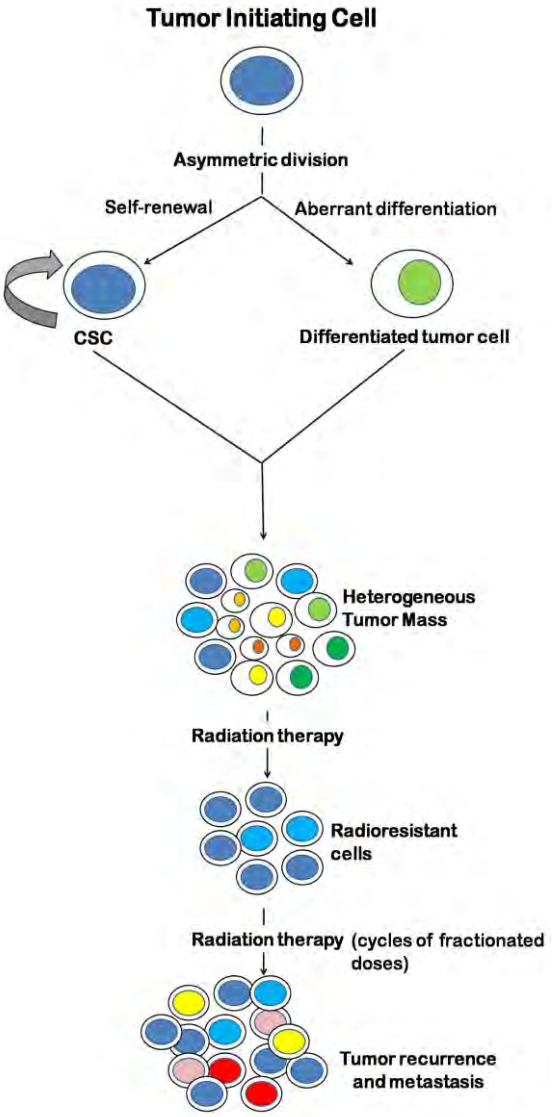

Figure 1: Role of CSCs in tumor radioresistance, recurrence and metastasis with relevance to cancer radiotherapy: Tumor initiating cell undergoes asymmetric cell division resulting in CSC and/or differentiated tumor cell. These cells further divide aberrantly to form heterogeneous tumor mass containing mixture of CSCs and non-CSCs at various stages of differentiation. After radiation therapy, radiosensitive cancer cells get killed and radioresistant cancer cells survive. The surviving cancer cells regrow and may result in highly resistant clones. These clones may cause recurrence and metastasis.

of cells expressing $CD44^{+}CD24^{-/low}$ were radioresistant (Phillips *et al*., 2006). The mechanism of radioresistance of CSCs was known to be associated with lower DNA double stranded breaks after radiation involving preferential activation of DNA damage check point kinases (CHK1, CHK2, ATM and Rad 17) leading to arrest of cell cycle for facilitated DNA repair (Bao *et al*., 2006, Rich *et al*., 2007; Yin and Glass 2011). Recently, in glioblastoma samples, the role of self-renewal gene (BMI-1) in mechanism of radioresistance was reported (Facchino *et al*., 2010). The authors demonstrated that overexpression of BMI-1 resulted in radio-resistance of CSCs and silencing of BMI-1 resulted in increased double strand breaks after irradiation (Facchino *et al*., 2010). In breast cancer, Wnt/beta-catenin signalling has a role in radioresistance and cell survival, resulting in tolerance of DNA damage (Woodward *et al*., 2007). A higher activity of free radical scavenging pathways in CSCs is another mechanism, resulting in lowering DNA damage after irradiation. This question was addressed in breast CSCs and these results showed that even after 10 Gy of gamma radiation, reactive oxygen species (ROS) generation was lower in MCF7 mammospheres compared to MCF7-monolayer cell cultures (Phillips *et al*., 2006). This line of evidence gets further supported by our recent finding of increased superoxide dismutase (SOD) activity in CSCs of human

lung adenocarcinoma (A549 cells), correlating with higher clonogenic survival of CSCs after radiation treatment of up to 6 Gy (unpublished data, personal communication).

Targeting CSCs for Radiosensitization during Cancer Radiotherapy

CSCs are known to play a critical role in radioresistance of tumors and hence, it is imperative to target them for enhanced tumor killing. To radiosensitize CSCs, several groups have targeted self-renewal signalling cascades in different tumors. Cox2 inhibitor (NS398) was used to target Akt signalling, which resulted in radiosensitising of the radioresistant oesophageal cells (Che *et al*., 2011). By silencing, T-cell factor-4 in Wnt signalling, radiosensitization was achieved in colorectal cancer cell lines (Kendziorra *et al*., 2011). In glioma CSCs, Notch signalling was targeted by blocking Notch1 or Notch2 ligands to radiosensitize the cells (McGowan *et al*., 2011; Wang *et al*., 2010). JAK/STAT signalling was also implicated in radioresistance of head and neck carcinoma CSCs and non-small cell lung carcinoma CSCs. Targeting these cells with STAT3 inhibitor, cucurbitacin, resulted in apoptosis and loss of tumorigenesis in xenograft mouse model (Chen *et al*., 2010; Hsu *et al*., 2011). TGF-β signalling gets activated in response to radiation, mediating its effect through Smad family and competes with Notch-ICD (intra cellular domain) protein (Masuda *et al*., 2005; Tian *et al*., 2009). Interaction of these pathways is responsible for maintenance of CSCs in tumors. Thus, targeting the molecules associated with the signalling events may offer novel therapeutic intervention for improved radiotherapy.

Some of the signalling molecules like PTEN, mTOR, CD23 and CD44 are

Table 1: Drugs used to target cancer stem cells to overcome radioresistance

Cancer Types	Targets	Drugs	References
Breast Cancer, Oesophageal cancer and Rectal cancer cells	Wnt/β-catenin pathway	NS 398 (COX2 inhibitor), LGK974	(Che *et al*., 2010) http://clinicaltrials.gov: NCT01351103
Glioma and medulloblastoma	Notch Signalling pathway	RO4929097	http://clinicaltrials.gov: NCT01122901
Small cell lung cancer	PI3-mTOR pathway	VS-5584	American Association of Cancer Research Annual Meeting; April, 2014
Lung cancer, Head and neck cancer	JAK/STAT signalling	Cucurbitacin I	(Chen *et al*., 2010; Hsu *et al*., 2011)
Breast cancer	Glutamate-cysteine ligase inhibitor	Buthioninesulfoximine	(Diehn *et al*., 2010)

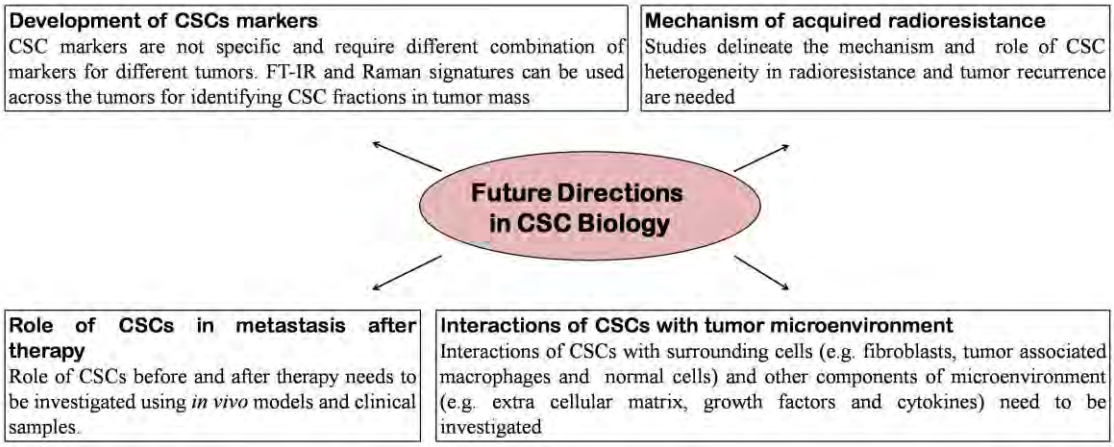

Figure 2: Future directions of cancer stem cell biology

differentially regulated in CSCs and normal stem cells, which can be exploited for targeted therapy of CSCs (Chen *et al*., 2013). Targeting the tumor suppressor PTEN in CSCss, sparing normal stem cells, was shown to be achieved in leukemia (Yilmaz *et al*., 2006). Recently, efforts were made to target glioblastoma CSCs using CD133 antibody tagged with gold nanorods for therapy using photoablation (Wang *et al*., 2011). The self renewal gene BMI-1 expressed in CSCs was also targeted in colon cancer in a mouse model (Kreso *et al*., 2014). Potential agents to target these signalling cascades of CSCs are currently in clinical trials (Table 1).

The other strategy evaluated to effectively kill CSCs using proton and heavy ion radiation like carbon ions are under consideration (Chang *et al*., 2010; Schlaff *et al*., 2014). Protons were observed to induce higher level of ROS and apoptotic death in CSCs in non-small lung carcinoma cell lines (Chang *et al*., 2010). The effect of carbon ion on CSCs was compared against X-rays, and the conclusion was that at the same dose, CSCs were enriched in xenograft tumors irradiated with X-rays compared to tumors treated with carbon ions (Cui *et al*., 2011). In the recent study, it was also shown that by targeting check point kinases and blocking ALDH1 activity in combination can radiaosensitize CSCs in head and neck cancer by photon or carbon ion radiation (Bertrand *et al*., 2014).

FUTURE DIRECTIONS

Even though substantial research has been made in the area of cancer stem cell biology, a more focussed research is required to understand the molecular interaction of CSCs with other cancer cells and components of tumor microenvironment. This would provide deeper understanding about role of CSCs in cancer radioresistance (Fig. 2). Characterization of novel biomarkers is very crucial to isolate CSC population. Furthermore, investigation of molecular

signaling mechanisms pertaining to radioresistance in CSC population may have significant implications in cancer therapy. Quantification of CSCs in tumor samples using sensitive, high throughput, reliable and economic techniques would be required to translate the knowledge gained in cancer stem cell biology to clinical level. Evaluation of CSCs in biopsy samples in cancer patients and their clinical correlation with tumor recurrence and metastasis may provide CSCbased prognostic markers in cancer therapy. It may be worth mentioning that better understating about exciting facts of cancer stem cell biology will benefit the cancer patients in coming days, which however, needs bridging the gap between laboratory and clinics.

CONFLICT OF INTEREST

The authors claim no conflict of interest.

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An Update on Cancer Prevention Approaches

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Majority of human cancers are caused, mediated and modified by environmental and lifestyle factors; and the multi-factorial, multi-step and multi-path process of carcinogenesis involves a series of genetic and epigenetic events. In spite of tremendous advancement in understanding of the molecular basis of cancer and identification of several environmental carcinogens, avoidance of exposure to carcinogens and early detection and/or successful treatment for most cancers have met with limited success. Based on the susceptibility to modulations of the multi-step process of carcinogenesis by a multitude of environmental compounds, lifestyle changes and host factors, and the demonstrated success of prevention of certain infectious diseases and cardiovascular events, cancer preventive interventions are receiving increasing attention. Several cancer preventive interventions such as vaccination, chemoprevention, weight control and lifestyle changes have been implemented. The current review focuses on several approaches and agents that have been scrutinized by way of randomized clinical trials in humans for their cancer prevention potential. Successful chemopreventive agents include selective oestrogen receptor modulators and aromatase inhibitors for breast cancer, the 5-α-reductase inhibitors for prostate cancer, non-steroidal anti-inflammatory drugs (NSAIDs) for colorectal lesions and vaccines for viruses that are associated with cervical and liver cancers. Several experimentally proven chemopreventive agents have been observed to lack efficacy with and without toxicity. In spite of numerous chemoprevention trials, the number of successful agents is rather small. Identifying novel approaches and chemopreventives holds tremendous potential for reducing the burden of cancer.

INTRODUCTION

Cancer comprises a multiple of diseases in which the cells proliferate autonomously without control, and accumulated abnormal cells spread to other parts of the body by invasion and/or distant metastasis via the blood and lymphatic systems. Cancer continues to be one of the major physical, social and economic burdens and public health threats worldwide and accounts for over 12% of deaths globally. The studies in migrant populations, changes in cancer incidence with time within same country and identical twins indicate that environment and lifestyle factors are major players in the causation of human cancer. The etiology of all cancers can be categorized into two main groups i.e. hereditary and environmental. Of the total, 5–10% of cancers are associated with inherited genetic aberrations; other 90–95% of

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cases are caused by exogenous/endogenous environmental factors.

The International Agency for Research on Cancer (IARC) has classified human carcinogens and recently completed reviewing human cancer sites associated with more than 100 carcinogenic agents (Cogliano *et al*., 2011). In brief, the list is given below:

- chemicals and mixtures e.g. aflatoxins, 4-aminobiphenyl, benzene, benzidine, coal tar pitch, ethylene oxide, formaldehyde, 2-naphthylamine, tobacco-specific nitrosamines, shale oils, soot, sulfur mustard, vinyl chloride, etc.
- several occupations e.g. productions of aluminium, auramine, coke, isopropyl alcohol, magenta, painting, rubber production industry, welding, etc.
- metals e.g. arsenic, beryllium, cadmium, chromium, nickel, etc.
- dusts and fibres e.g. asbestos, dust from leather, silica, wood, etc.
- radiations e.g. all types of ionizing radiations, UV/solar radiations
- biological agents e.g. EBV, HBV, HIV1, highly oncogenic HPVs, HTLV1, Kaposi sarcoma herpes virus, parasites such as liver flukes and schistosoma and bacteria such as *Helicobacter pylori*, etc.
- personal habits e.g. smoking/smokeless tobacco use, alcoholic beverages, use of areca nut, betel quid with/without tobacco, indoor emissions from household combustion, consumption of salted fish, etc.
- pharmaceuticals e.g. several anti-cancer agents, immunosuppressants, hormonal preparations, etc. (Cogliano *et al.*, 2011).

Nevertheless, several common human cancers have not been associated with identified causative agents and the quest for causative factors continues.

Although dose and duration of exposure to exogenous/endogenous carcinogen(s) are some of the determining factors, these aspects are not sufficient to explain exposure-related outcome as majority of cancers result from complex interactions between environmental exposure(s) and genetic/acquired susceptibility or protective host factors. Response to carcinogen exposure may further be modulated by other risk-enhancing or protective factors such as diet, tobacco and alcohol, physical activity, obesity leading to associations with cancer.

Carcinogenesis

Carcinogenesis refers to chemical/physical/biological agent(s)-mediated etiologic pathway that leads to cancer. It is a complex, multi-factorial, multi-step, multi-path process characterized by at least three stages viz., initiation, promotion and progression (Fig.1). Initiation is an irreversible event which begins when the cells in normal tissues are exposed to carcinogen and the genomic DNA undergoes damage and subsequent fixation of the damage. In the promotion process, initiated

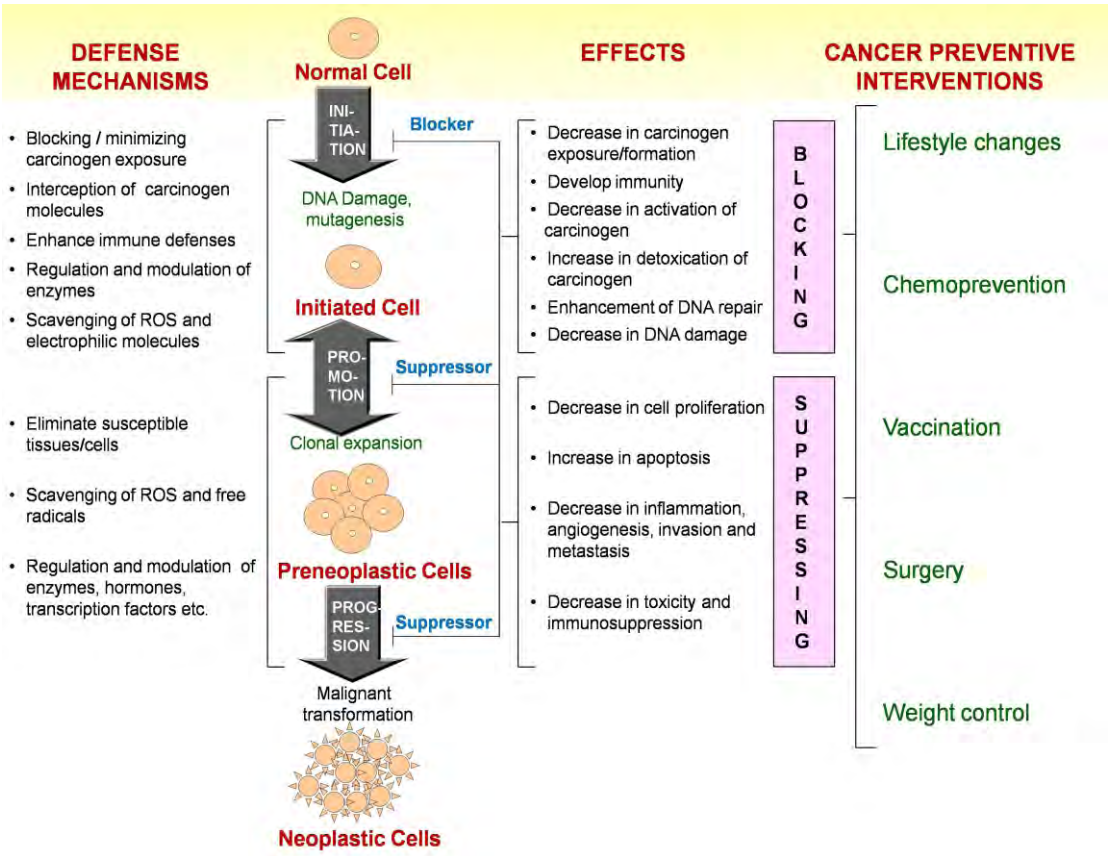

Figure 1: Schematic representation of the multi-step process of carcinogenesis, steps defining cancer preventive interventions and their effects, and observed defense mechanism(s).

cells expand to form an actively proliferating multi-cellular pre-malignant tumor cell population; while progression is an irreversible process producing a new clone of tumor cells with increased proliferative capacity, invasiveness and metastatic potential.

Among several models of carcinogenesis, Knudson (1971) proposed a 'two-hit' model requiring a mutation in both copies of a gene resulting in cancer. Expansion of this concept by Vogelstein and colleagues emphasized that cancer is ultimately a disease of damaged DNA comprising of a series of genetic mutations that lead to the transformation of normal cells to cancerous cells (Vogelstein and Kinzler, 2004). The genetic mutations include inactivation of tumor suppressor genes and/or activation of oncogenes. Further expansion of the concept by Hanahan and Weinberg (2011) proposed hallmark events at the cellular level that lead to cell transformation. The hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. Underlying these hallmarks are genome

instability and inflammation, which fosters multiple hallmark functions leading to cell transformation and imparting the ability to invade and metastasize.

In spite of tremendous advancements in understanding the molecular basis of cancer, early detection and/or successful treatment approaches for most cancers have met with limited success. It is expected that the number of cancer-related deaths may double in the next 50 years and global cases of cancer will rise to 15 million new cases by 2020, when the world population reaches 7.5 billion. Generally, patients with metastatic cancer do not successfully respond to even the most advanced treatment methods, and often their lives are not saved. While in patients with less advanced cancer, treatment extracts tremendous social and economic devastation. Moreover, an increasing trend of chemo-/radio-resistance and recurrence of tumor results in limitations in the fight against cancer.

In this context healthy aging and disease prevention by preventive interventions is increasingly a subject of academic and public interest and research. The approach of cancer prevention by avoiding/minimizing exposure to proven carcinogens, and/or altering the metabolism of carcinogen(s), and/or pursuing lifestyle or dietary changes to modify effect of cancer causing factors or genetic predispositions, and/or medical interventions (chemoprevention), and/or prophylactic resection of high-risk organs in certain germline mutation carriers is considered to be an alternative, probably more realistic, cost-effective and a fundamental strategy. This is based on the experience that preventable illnesses make up ~80% of the burden of illnesses and 90% of all healthcare costs, and saves us from undergoing sufferings and discomfort.

The rationale for prevention approaches are based on:

- process of carcinogenesis involves a series of genetic and epigenetic events (multi-step, multi-path), and that many of these events are susceptible to modulation by variety of environmental compounds, lifestyle changes and host factors, and
- prevention approaches have been demonstrated to be successful in other environmental diseases such as certain infectious diseases (vaccines) and cardiovascular events (treating risk factors with stents, statins, lifestyle changes).

The concept of prevention is best defined in the context of levels traditionally called primary, secondary and tertiary prevention.

Primary Prevention

Primary prevention is an important means to improve public health, and it is by far the most cost-effective and sustainable intervention for reducing mortality and disability, by reducing the incidence of cancer globally. Primary

prevention of human cancer can be accomplished in two ways:

- avoiding the introduction of carcinogenic agents into the environment, and
- eliminating or drastically reducing exposure to carcinogenic agents that are already present in our environment

The first approach is theoretically possible but practically it has proved to be difficult. The second approach involves actions aimed at reducing or eliminating exposure to carcinogens, and/or enhancement of resistance to the effects of exposure to causative agents (e.g. vaccination). All these approaches are suitable for application in general public.

Elimination of carcinogen/avoidance or minimization of exposure can probably be achieved by improving technology, and/or replacement of agent by less toxic or non-chemical means. Avoidance or minimization of exposure can also be achieved by implementing control measures resulting in a decrease in exposure conditions such as concentration, duration, time, frequency and also reducing exposure to the number of persons. This can be facilitated by increasing awareness, reducing workplace limits, improving compliance with exposure limits by legislation, regulation and policies. This is likely to be achieved in occupational and environmental exposures. In addition, motivating individual members, institutions/organizations and society for changes, in attitudes and behaviors are also important in exposure control and risk reduction. Although possible, it has proved difficult, possibly due to addiction, especially with respect to societal exposures e.g. smokeless tobacco/tobacco smoke, alcohol, etc.

Although primary prevention of occupational carcinogen(s)-induced cancers appears to be simple and logical, reductions in cancer rates are not easily documentable in quantitative terms (Tomatis *et al.*, 1997) perhaps due to the following reasons:

- Few follow-up studies designed to determine whether cancer rates actually declined as a result of implementation of defined preventive measures.
- Most reports predicting decline in cancer risk are based on assumptions about exposure-response curves and not on actual observations on changes in risk after exposure reduction.
- The time that must elapse after intervention, before a reduction in cancer risk (that may vary depending on the dose and duration of exposure) can be observed, may be one of the reasons for the absence of data.
- The multi-factorial origin of most tumors makes it particularly difficult to measure the role and quantify the contribution of a single agent.
- Estimates of attributable risks are largely based on unverified assumptions and hence most assessments of the percentage

of cases that could be avoided by intervening on single factors are uncertain.

- The concept of prevention is further complicated because 'attributable risk' is taken as the proportion of all cases of the disease caused by individual exposure.
- Research funding may be more difficult to obtain for (long-term) studies to demonstrate the effectiveness of primary preventive actions.

In spite of convincing evidence for their causal association with cancer for more than 100 environmental carcinogenic agents, their elimination from the environment has proved to be difficult due to lifestyle factors and modern developments. Overall there is limited success in elimination of carcinogen, and/or avoidance of carcinogen exposure. In addition, specific causative agents have not been identified for several cancers, partly because of the limitations of available experimental/epidemiological studies, and hence primary prevention is not achievable for them. This approach has demonstrated some success and feasibility especially in preventing societal, occupational and environmental exposure-related, as well as some biological agent-induced cancers. Some of the examples are indicated as follows:

- The decreasing risk of cancer in British male doctors who quit smoking provides strong evidence that the elimination of exposure results in reduction of risk (Doll *et al.*, 1994). Decrease in lung cancer rates with exposure reduction in terms of number of cigarettes per day, or duration of smoking and time since stopping smoking (Lubin, 1984), and decreasing mortality from lung cancer in males from younger cohorts in the western world, have been linked to the decreasing proportion of smokers among the young (Coleman *et al.*, 1993). Lung cancer death rates in USA have mirrored smoking patterns i.e. increase in smoking followed by dramatic increase in lung cancer death rates and, more recently, decrease in smoking followed by decrease in lung cancer death rates in men (Jemal *et al.*, 2001).
- Banning production and use of carcinogenic aromatic amines has resulted in reduction of bladder cancer among dye workers after the elimination of exposure to aromatic amines (Swerdlow, 1990).
- A significant reduction in incidence and mortality for gastric cancer has been attributed to the elimination of environmental carcinogens, and/or the improvement in food preservation techniques (Hwang *et al.*, 1994).
- Initial observation based evidence suggest the success of vaccines against specific causative biological agents such as HBV, HPVs (Chang *et al.*, 1997; Frazer, 2004).
- Reduced melanoma after regular sunscreen use has been reported based on

observations from randomized follow up trial (Green *et al.*, 2011). This result has further been complimented by the effect of sunscreen on the response of melanocytes *in vivo* to ultraviolet radiation (Hacker *et al.*, 2013).

Creating awareness and implementing appropriate preventive measures may help us in decreasing the incidence of region-specific cancers such as Kangri Cancer (due to prolonged exposure to heat) in Kashmir, Sari and Dhoti Cancer (Chronic friction) in Maharashtra, and tongue/mouth cancer due to sharp tooth.

In traditional primary cancer prevention approaches, research on healthy lifestyles e.g. strategies to reduce unhealthy behaviors, and identification and elimination/avoidance of environmental risk factors receive major emphasis. While complementary cancer prevention activities may include,

- screening of populations for genetic risk factors and genetic counseling of individual with genetic risk
- research to develop new, more sensitive and specific biomarkers for early detection of cancer
- screening of populations for early detection of certain cancers

Screening is the early detection of disease, precursors to disease, or susceptibility to disease, in individuals who do not show any signs of disease.

Screening populations for genetic risk factors

Hereditary cancer is cancer risk that is inherited or passed on in a family. Hereditary cancer results from an abnormal alteration in a single gene and 5–10% of all cancers are considered to be hereditary, e.g. breast-ovarian cancer syndrome, familial adenomatous polyposis (FAP), familial melanoma, hereditary non-polyposis colorectal cancer (HNPCC), multiple endocrine neoplasia (MEN), Von Hippel Lindau (VHL) syndrome, xeroderma pigmentosum, and hereditary diffuse gastric cancer (HDGC), etc.

Compared to cancers arising in the general population, individuals with a major inherited predisposition to cancer are born with inherited (e.g. germline) mutations in genes involved in cancer causation. Since the heritable component of some cancer predisposition has been linked to mutations in specific genes, clinical interventions have been formulated for mutation carriers within affected families. Surgery represents the primary approach to cancer prevention for carriers of mutations in genes associated with high penetrance cancer syndromes, such as MEN, FAP, HNPCC and HDGC. Prophylactic resection of high-risk organs in certain germline mutation carriers although radical, may be recommended. However, standard prevention approach e.g. colostomy can reduce colorectal cancer risk in FAP patients who have adenomatous polyposis coli

mutations (Chau and Cunningham, 2002; Steinbach *et al.*, 2000) and bilateral mastectomy (Meijers-Heijboer *et al.*, 2001) and oophorectomy (Haber, 2002; Metcalfe, 2009) can reduce breast cancer risk as well as breast and ovarian cancer risk in BRCA1/BRCA2 mutation carriers. Several studies have provided evidence that genetic counseling and testing increased surveillance and led to risk-reducing operations, which resulted in a diagnosis of early-stage tumors in patients with BRCA1 and BRCA2 mutations (Scheuer *et al.*, 2002).

Several studies have employed alternative prevention approaches in carriers of gene mutations, e.g. tamoxifen in individuals with germline BRCA2 mutation. Tamoxifen reduced breast cancer incidence among healthy BRCA2 carriers by 62%, similar to the reduction in incidence of ER positive breast cancer among all women in the breast cancer prevention trial (King *et al.*, 2001). In contrast, tamoxifen use beginning at the age of 35 years or older did not reduce breast cancer incidence among healthy women with inherited BRCA1 mutations.

Screening for early detection of certain cancers

Screening for early detection of several cancers in asymptomatic individual to reduce mortality and morbidity has been employed. Beyond the potential of avoiding death, screening may reduce cancer morbidity since treatment of earlier stage cancers is often less aggressive than that for more advanced-stage cancers. These interventions are often directed to entire populations or large and easily identifiable groups within the population. Several simple and/or advanced methods/markers have been employed for early detection of breast cancer (e.g. Mammography, Breast self-examination), cervical cancer (Pap smear, VIA), colorectal cancer (Fecal occult blood testing, Sigmoidoscopy, Colonoscopy), prostate cancer (PSA, Digital rectal examination) and several other cancers. The approach of early detection of cancer has demonstrated success in reducing cancer mortality (Christopherson *et al.*, 1976; Mandel *et al.*, 1993; Shapiro *et al.*, 1982; Shastri *et al.*, 2014); however, screening use remains low in resource-poor countries. This approach may also facilitate enrollment of subjects for secondary or tertiary chemoprevention trial ultimately leading to a reduction in mortality and morbidity due to cancer.

Secondary Prevention

In secondary prevention targets are specific risks in closely defined high-risk subjects rather than general populations, and intervention is undertaken to prevent the consequences of carcinogen exposure, and preclinical disease.

Tertiary Prevention

In tertiary prevention, aim is to prevent or control the symptoms and morbidity due to cancer or cancer therapy, and prevent recurrence of pre-existing cancer or a subsequent different cancer (second primary).

Preventive interventions

Several preventive interventions such as chemoprevention, vaccination, weight control and lifestyle changes (avoiding/minimizing exposures, physical exercise, eating healthy) have been employed (Fig.1) and extensive experience and literature on various aspects of 'chemoprevention' and 'vaccination' have accrued.

Chemoprevention

Chemoprevention of cancer refers to intervention with natural or synthetic compounds to retard, block or reverse the process of carcinogenesis ultimately leading to prevention of pre-neoplastic and/or clinically detectable cancer and/or recurrence of cancer. It is a 'prescription' approach and forms an adjunct to other cancer control and prevention approaches.

Identification of environmental chemo-preventive agents

Considering the limited scope of the present update only a very brief summary of this aspect has been covered. Putative chemopreventive agents are subjected to rigorous evaluations employing a series of *in vitro* and *in vivo* experimental model systems (Table 1).

Studies employing *in vitro* and/or *in vivo* animal models have contributed significantly in identification of a number of environmental chemopreventive agents and helped in understanding the complexity of gene-environmental interactions (Patel *et al.*, 2007). After extensive evaluation of preclinical efficacy and safety of an agent, further evaluation in appropriate clinical trials is

Table 1: Experimental models and end points employed in identification of environmental chemopreventive agents

***In vitro* assays**	**End points**
Bacteria, mammalian cells, tissues, organ cultures, cancer cell lines, cell-free extracts or biochemical reactions	**Inhibition of carcinogen/mutagen-induced effects –** mutations, chromosomal aberrations, clastogenic effects, DNA adducts and free-radical formation, DNA strand breaks, levels and activity of metabolic and repair enzymes **Inhibition of cell proliferation –** colony growth in soft agar, transformed cell foci, alterations in response to a known stimuli **Enhancement of cell differentiation and apoptosis**
***In vivo* assays**	**End points**
Normal/genetically engineered rodents and other models	Decrease in incidence and/or multiplicity of carcinogen-induced or spontaneous tumors or inhibition of carcinogen-induced premalignant lesions or markers or pathways. Increase in tumor latency period

undertaken.

Clinical trials are prospective biomedical studies on human subjects to answer specific questions about interventions (drugs/ treatments/vaccines/devices or new ways of using known interventions) generating safety and efficacy data. Preventive interventions initially enroll volunteers and/or patients in pilot studies and subsequently conduct progressively larger scale comparative studies. Clinical trials can vary in size involving single or multiple research entities in one or more countries. The goals, enrollment of the study population, study protocols, acceptable level of toxicity, etc. in the design of chemoprevention trials are different when compared to therapeutic oncology drug treatment trials (Table 2).

Interventions demonstrating chemo-preventive efficacy

a) Earlier reports demonstrated that anti-estrogens play an important role in preventing breast cancer development (Jordan *et al*., 1980). Tamoxifen, a selective estrogen receptor modulating agent (SERM), was discovered as an anti-estrogen compound and has been used for over 30 years in patients with early stage breast cancer as adjuvant therapy to prevent breast cancer recurrence; and in those with metastatic breast cancer to slow down its growth (Fisher *et al*., 1989; 1998).

The breast cancer prevention trial (BCPT) and follow up study of tamoxifen against raloxifene (STAR), two randomized, double blind, placebo controlled trials, enrolled a total of more than 32,000 women (35 years or older) at risk of breast cancer (Fisher *et al*., 1998; Vogel *et al*., 2006) to study whether tamoxifen can reduce the risk of developing breast cancer. The BCPT trial demonstrated conclusively that 20 mg/day of tamoxifen reduced the incidence of invasive breast cancer by 45%; ductal carcinoma was reduced by 48% compared with women on placebo; at least a 1.66% risk of invasive breast cancer over 5 years. Women, who took tamoxifen, had a significant increase (2.4 fold) in the risk of developing endometrial carcinoma and an increase in venous thromboembolic events (Fisher *et al*., 1998). The STAR trial compared tamoxifen and raloxifene for preventing breast cancer in post-menopausal women. Both the agents exhibited ~ 50% reduction in breast cancers and raloxifene showed fewer adverse effects including uterine cancers, cases of thrombosis and hot flashes) (Vogel *et al*., 2006). These trials have demonstrated efficacy of either SERM for breast cancer prevention and also suggested potential for improved safety in the iterative generation of agents.

In the worldwide adjuvant tamoxifen:

Table 2: Design of prevention trials vs therapeutic treatment trials

	Prevention Trials	Therapeutic Treatment Trials
Goals	**Cancer prevention**	**Cancer treatment**
	decrease in incidence and mortality	increase in cure or remission rates
	prevent/ameliorate precancerous lesions/biomarkers that serve as surrogates of risk	decrease in mortality and morbidity
	prevent second primary tumor	improvement in survival and/or efficacy against an established surrogate end point
Study Population	**Subjects without cancer** (asymptomatic, ostensibly healthy subjects)	**Cancer patients** (diagnosis confirmed before therapy)
	general population	
	high-risk population	
	persons with precancerous lesions	
	cancer patients	
	small to large-scale	small to moderate
Toxicity of Agent	**Mild to moderate – acceptable**	**Moderate to severe – acceptable**
Study Protocol	**Design**	**Design**
	intervention vs placebo	therapy vs placebo
	intervention A vs intervention B Vs intervention AB vs placebo	therapy A vs therapy B
		therapy A vs therapy B vs therapy C
	pilot study usually required	pilot study rarely needed
	placebo run-in is useful	placebo run in is inappropriate
	study may require 5-10 years or more of intervention and follow up	study length may be short for aggressive cancers, longer for slow growing cancers/adjuvant studies
	adherence to protocol may be difficult to maintain (subject-dependent)	adherence to protocol easier to maintain (physician-dependent)
Basis	**Based on cellular and molecular insights**	**Based on symptoms and loss of normal function**

longer against shorter (ATLAS) trial, 12,894 women with early breast cancer who had completed 5 years of treatment with tamoxifen were randomly allocated to continue tamoxifen for 10 years or stop at 5 years (open control). Results demonstrated that women with ER +ve disease, continuing tamoxifen for 10 years rather than stopping at 5 years produced a further reduction in recurrence and mortality particularly after year 10 (Davies *et al*., 2013).

Following their successful implementation for the treatment of metastatic breast cancer, the third-generation aromatase inhibitors (anastrozole, letrozole, exemestane) have now become the standard adjuvant endocrine treatment for postmenopausal estrogen-receptor-positive breast cancers (Lonning and Eikesdal, 2013). In a randomized, placebo-controlled, double blind trial, 4560 women (median age 62.5 years, median Gail risk scores 2.3%) were assigned to either exemestane or placebo. At a median follow-up of 35 months, 11 invasive breast cancers were detected in those given exemestane, and 32 in those given placebos, with a 65% relative reduction in the annual incidence of invasive breast cancer, and no serious toxic effects (Goss *et al*., 2011).

b) The high-risk human papilloma virus (HPV) is the etiologic agent associated with cervical cancers in females, penile and anal cancers in males, and 5–10% oropharygeal cancers in both males and females (Dunne *et al*., 2012). A causative relationship between high-risk HPVs e.g. HPV16 and HPV18 infection and cervical cancer has been established (Boshart *et al*., 1984; Durst *et al*., 1983). Approximately 70% of cervical cancers are caused by HPV16 and HPV18. Quadrivalent and bivalent vaccines against high-risk HPV types HPV16 and HPV18, and/or HPV6 and HPV11 have shown 95–100% effectiveness at preventing the cervical cancer precursor lesion (cervical intraepithelial neoplasia grade 3 or greater) and 100% effective at preventing cervical adenocarcinoma *in situ*. When considering the entire cohort tested, including those with prior HPV infection, the level of protection conferred is highly variable with 12–46% protection from cervical intraepithelial neoplasia grade 3 or higher, and 28–83% protection from cervical adenocarcinoma *in situ* (Garland *et al.*, 2007; Group FIS, 2007; Lehtinen *et al.*, 2012). Two HPV vaccines i.e. Gardasil (quadrivalent vaccine against 4 HPV types – 6, 11, 16 and 18 from Merck) and Cervarix (bivalent vaccine against HPV types 16 and 18 from Glaxo SmithKline) have been approved by Food and Drug Administration, USA are globally in use.

Another cancer with the potential for vaccine-based prevention is hepatocellular carcinoma (HCC). In Taiwan when HBV vaccination was implemented the rates of childhood HCC decreased in children (Age 6–14 years) from 0.70 cases per 100,000 in 1981–1986 to 0.36 cases per 100,000 in 1990–1994 (Chang *et al.*, 1997). The impact of HBV vaccination on HCC occurrence will be realized after about 20 years of vaccination (Wong and Chan, 2012).

c) Multiple lines of evidence suggest non-steroidal anti-inflammatory drugs (NSAIDs) are active in colorectal adenoma and cancer prevention (Chan *et al.*, 2012; Giardiello *et al.*, 1993). Observational evidence for the association between NSAIDs and colorectal adenomas indicated that the relative risk of colorectal adenomas was 0.57 with regular use of any NSAID; and for non-aspirin NSAIDs the effect was somewhat smaller with a relative risk of 0.7. Colorectal adenomas are considered as precursor lesions to cancer. NSAIDs-mediated adenoma risk reduction of 22–53% puts NSAIDs at the forefront of agents of interest in colorectal cancer prevention (Gill and Sinicrope, 2005; Rostom *et al.*, 2007). At low levels of cardiovascular risk, the benefit of aspirin for colorectal adenoma prevention assumes increased importance in the balance against the complications of aspirin/NSAID use. After 5 years follow up in the Physician's Health Study with participants randomized to 325 mg of aspirin every other day versus placebo, the point estimate for colorectal polyps and *in situ* cancer was 0.88 (Gann *et al.*, 1993). In the Women's Health Study, 100 mg of aspirin every other day was compared with placebo and after 10 years, the relative risk of colon polyps was 0.97 (Cook *et al.*, 2005). Although the doses tested may be suboptimal for colorectal adenoma prevention, concern about adverse events with higher doses is justified. A recent, notable success was reported from evaluating a longer-term follow-up study of Colorectal Adenoma/Carcinoma Prevention Program (CAPP2), evaluating aspirin and resistant starch for the prevention of adenomas and carcinomas in Lynch syndrome patients. Long-term follow-up demonstrated a significant increase in time-to-first colorectal cancer occurrence in those who took aspirin for at least two years (Burn *et al.*, 2011). A recent review presenting analysis of available evidence from studies and clinical trials suggests that prophylactic aspirin use in a general population for a minimum of 5 years at 75–325 mg/day appears to have favorable benefit-harm profile. For average risk individuals aged 50–65 years taking

aspirin for 10 years, a relative reduction of between 7% (women) and 9% (men) in the number of cancer, myocardial infarction or stroke over a 15 year period and an overall 4% relative reduction in all deaths over a 20 year period was reported (Cuzick *et al*., 2014).

With the development of selective COX-2 inhibitors (coxibs), the option of blocking the inducible form of COX offered an attractive opportunity. Three randomized controlled trials of COX-2 inhibitors confirmed a significant (28%) reduction of adenoma risk (Arber *et al.*, 2006; Baron *et al.*, 2006; Bertagnolli *et al.*, 2006; Steinbach *et al.*, 2000). Adverse effects included increase (2.6 fold) in cardiovascular events for a celecoxib dose of 400 mg and 3.4 fold for a dose of 800 mg. In addition to the proof-of-principle established by the results, coxibs may find a role in individuals at high risk of colon cancer with low cardiovascular risk. This scenario should apply to young patients diagnosed with familial adenomatous polyposis (FAP). A subsequent study of children with FAP found that celecoxib at a dosage of 16 mg/kg per day for 3 months was well tolerated and reduced the number of colorectal polyps by 44% (Lynch *et al.*, 2010).

Another successful chemoprevention trial was reported a few years ago, using the combination of difluromethyl ornithine (DFMO, 500 mg/day) and sulindac (150 mg/day) to prevent sporadic colorectal adenomas (Meyskens *et al.*, 2008). Individuals receiving this intervention experienced a 70% reduction in colorectal adenomas in contrast to participants assigned to the placebo arm of the trial, where 41% developed adenomas over 3 years (Meyskens *et al*., 2008). This trial utilized low doses of both DFMO and sulindac, thus limiting the potential toxicities associated with the medications. This approach allows for the targeting of multiple aspects of a single pathway (Gerner and Meyskens, 2009).

d) Melanoma and non-melanoma skin cancers are among the most prevalent cancers in human. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are non-melanoma skin cancers and actinic keratosis (AK) is a precancerous lesion that may progress to SCC if left untreated. Several methods of treating AK to prevent skin cancers are used based on demonstration of their efficacy in clinical trials. Actinic keratosis has been treated successfully by surgical removal of the lesion through cryosurgery or laser surgery, especially when the number of lesions is limited (Thai *et al.*, 2004). The other successful approach has been medical therapy wherein a chemical (e.g. chemical peeling) or medicated creams, gels and solutions are effective by

themselves or in combination with surgery (Berlin and Rigel, 2008; Jorizzo *et al.*, 2006). Medical therapy has advantages in being able to treat large areas with multiple lesions. Based on their success in several clinical trials five medications have been approved by FDA for the treatment of AK. These are topical 5-fluorouracil (5-FU) 0.5–5% (ointment or liquid) (Gupta *et al.*, 2005; Kurwa *et al.*, 1999; Loven *et al.*, 2002; Tutrone *et al.*, 2003a); topical imiquimod 2.5%, 3.75% and 5% (cream) (Gupta *et al.*, 2005; Hanke *et al.*, 2010; Stockfleth *et al.*, 2007; Ulrich *et al.*, 2006; 2007); topical diclofenac sodium 3% gel (Berlin and Rigel, 2008; Tutrone *et al.*, 2003b); photodynamic therapy (PDT) wherein light sensitizing compound such as delta-aminolevulinic acid is applied topically followed by exposure to light with appropriate wavelength that results in selective killing of dysplastic cells (Kaufmann *et al.*, 2008; Kurwa *et al.*, 1999); and topical ingenolmebutate, 0.015 or 0.05% gel (Lebwohl *et al.*, 2012; 2013; Rosen *et al.*, 2012). In four randomized, double-blind, and placebo-controlled studies, patients with AKs were randomized either to self-applied ingenolmebutate or placebo for 2–3 days. By 57th day, 42% of the patients with facial or scalp AKs who had received treatment experienced complete clearance of AKs compared with 3.7% in the placebo arm. Similarly, 34.1% of the participants with AKs on their trunks or extremities experienced complete clearance, whereas only 4.7% of those in the placebo arm had similar results (Lebwohl *et al.*, 2012). Amongst the agents employed ingenolmebutate appears to be more effective due to the shorter time of treatment.

e) In a community-based pragmatic trial of sunscreen (SPF 15+) to prevent skin cancer in Queensland, Australia; 1621 randomly selected residents of Nambour (Age 25–75) were randomly assigned to daily or discretionary sunscreen application to the head and arms for 4 years and participants were observed for 10 years. The reduction in invasive melanoma was substantial (n = 3 in active vs. n = 11 in the control group). Findings suggest the general preventability of melanoma after the regular application of broad-spectrum sunscreen (Green *et al.*, 2011).

f) The use of Bacilli-Calmette-Guerin (BCG) for treatment and prophylaxis of carcinoma *in situ* of the urinary bladder, and for prophylaxis of primary or recurrent stage Ta and/or T1 papillary tumors after transurethral resection (Sylvester, 2011), and use of valrubicin in BCG-refractory carcinoma *in situ* of the urinary bladder in patients for whom

immediate cystectomy would be associated with unacceptable morbidity or mortality have been reported (Steinberg *et al.*, 2000). Similarly, use of photofrin plus photodynamic therapy developed largely as adjuvant therapies for treatment of pre-invasive neoplastic lesion and for esophageal dysplasia have also been approved (Davila, 2011; Overholt *et al.*, 2005).

g) In a study involving the use of celecoxib for prevention of lung cancer in former smokers, emerging evidence shows that long-term use of celecoxib is associated with significant cardiovascular risk, and hence the study was suspended. Following reopening of the study, impressive response to celecoxib in a subset of patients was observed. As the study used small number of patients, it was difficult to generalize the results with confidence (Mao *et al.*, 2011). Based on this and other studies, celecoxib and other similar agents may need further evaluation as a chemopreventive in former smokers with high-risk lesions, and/or in individuals with high risk of developing colon cancer and low cardiovascular risk.

h) The androgen receptor blockers continue to be of interest for the prevention of prostate cancer. Finasteride that lowers the concentration of dihydrotestosterone by blocking 5α-reductase type 2, was tested in prostate cancer prevention trial (PCPT). In the phase III design, 18,882 men were randomized to receive finasteride versus placebo (Foley and Kirby, 2003). There was a reduction in prostate cancer prevalence of the order of 25% during a 7 year follow-up period, but apparently also an increase in high-grade tumors (Thompson *et al.*, 2003). Subsequent careful analysis showed that the excess high-grade tumors were probably due to biopsy artifacts (Logothetis and Schellhammer, 2008; Lucia *et al.*, 2007; Redman *et al.*, 2008). A similar reduction in prostate cancer has been reported with dutasteride – a dual 5α-reductase inhibitor (type 1 and 2) in a placebo-controlled trial, wherein 6729 men showed 23% reduction in the risk of prostate cancer at the end of 4 years with no apparent increase in high-grade cancer (Andriole *et al.*, 2010).

i) In a Physician Health Study, PHS-II, 14,641 male physicians were randomly assigned to receive either a daily multivitamin supplement or a placebo for a median of 11 years. Multivitamin supplements were associated with an 8% relative decrease in cancer incidence. The overall reduction in cancer risk was more pronounced in men diagnosed with cancer before the study began than those with no history of cancer. The study suggested that the small benefit of multivitamins in reducing overall cancer incidence largely stemmed from the prevention of second

primary cancer (Gaziano *et al.*, 2012).

Interventions demonstrating lack of chemopreventive efficacy and/or toxicity

a) Earlier studies (Hong *et al.*, 1986; 1990; Meyskens *et al.*, 1994) with high doses of retinoids for its effects on oral intraepithelial neoplasia (IEN) and cervical IEN (Hong *et al.*, 1986; Meyskens *et al.*, 1994) suggest a protective role by prevention of secondary head and neck malignancies (Hong *et al.*, 1990). Based on these initial observations of protective effects of β-carotene, the alpha tocopherol and β-carotene (ATBC) prevention study was undertaken. In this study > 29,000 Finnish male workers (age 50–60 years) were randomized to one of the four groups: α-tocopherol (vitamin E), β-carotene (a precursor of vitamin A), both α-tocopherol and β-carotene, or placebo with a 5–8 year follow-up. Men who took β-carotene alone or in combination with vitamin E had an 18% increased incidence of lung cancer and an 8% increase in overall mortality; whereas vitamin E alone had no effect (ATBC Prevention Group, 1994).

   The CARET trial that was a double-blind, placebo-controlled study enrolled > 18000 male and female smokers, former smokers and asbestos-exposed workers to study the effects of β-carotene and retinyl palmitate (vitamin E) or placebo on lung cancer and cardiovascular disease. The trial was stopped early after an interim analysis showed a 28% increase in lung cancer incidence and a 17% increase in overall mortality in the treatment group (Omenn *et al.*, 1996). Subsequent trials employing lower/less toxic doses of retinoids evaluating its effect on cancer and other endpoints have been negative (Decensi *et al.*, 2000; Lippman *et al.*, 2001). Thus retinoids have not been successful in reducing the cancer risk.

b) In selenium and vitamin E cancer prevention trial (SELECT), > 35,000 men (Caucasians aged > 55 years; African Americans aged > 50 years) from US, Peurto Rico and Canada were randomized to treatment with vitamin E and selenium together or placebo. The study was terminated earlier due to lack of efficacy in interim analysis (Klein *et al.*, 2003). Continued follow-up of study participants showed a 17% increase in prostate cancer risk in healthy men receiving vitamin E alone (Klein *et al.*, 2011).

c) The results of the Physicians' Health Study II demonstrated that supplementation with vitamin E and/or vitamin C had no benefit compared with placebo in preventing either prostate cancer or total cancer incidences (Gaziano *et al.*, 2009).

d) The results of the Women's Antioxidant Cardiovascular Study indicated that,

compared with placebo, supplementation with vitamin C, vitamin E or β-carotene was not efficacious in reducing the total cancer incidence (Lin *et al.*, 2009). In the same study, daily supplements containing folic acid, vitamin B6 and vitamin B12 as compared to placebo was not efficacious in reducing the overall risk of developing cancer (Zhang *et al.*, 2008). An exploratory analysis of pooled data from two Norwegian randomized controlled trials showed an increase in both cancer incidence and cancer death in patients treated with folic acid and vitamin B12 versus those receiving placebo or vitamin B6 alone (Ebbing *et al.*, 2009).

e) Evidence on the efficacy of vitamin D supplements (400–1100 IU daily) with or without calcium in preventing cancer incidence is available as a secondary endpoint from randomized controlled trials. All the trials showed lack of efficacy (Avenell *et al.*, 2012; Chung *et al.*, 2011).

A recent search of 'clinicaltrials.gov' showed a list of 280 on-going/completed chemoprevention trials. The majority of the chemopreventive agents fail to achieve endpoint results in randomized clinical trial settings due to lack of efficacy and/or unexpected toxicity. Most of the agents demonstrating preventive effects in experimental models have failed to exhibit chemopreventive effects in clinical trials. These failures can broadly be attributed to lack of (a) ability to replicate the conditions of human exposure (route, dose, sequence and frequency, duration etc.) and other host factors in animal models; and (b) knowledge about the mechanism(s) of action and toxicity of the agent on normal physiological processes in different organ systems (Patel *et al.*, 2007).

In spite of numerous chemoprevention trials, the number of successful or approved agents is rather small (Table 3). This is partly due to the challenges and barriers including (a) choice of cohorts – including difficulties in identifying and recruiting participants, which influences the outcome of the trial by affecting time lines, statistical power and adherence; (b) agent(s) with powerful efficacy in preclinical studies, selection of optimal doses, route, duration and frequency and toxicities; and (c) endpoint including the long latency period to cancer endpoints, selection of biomarkers as surrogates and accessibility to the target organ(s). The difficulties lead to very high costs, extended follow up periods and complexity in assessment of risk-benefit of the cancer risk reducing drugs (Table 3).

There is dire need to (a) improve existing experimental models or develop new experimental models/approaches to achieve better replication of human host factors and/or exposure conditions; (b) generate adequate information about the mechanism(s) of action of observed chemopreventive efficacy and/or toxicity; (c) study, understand and if feasible exploit the role of diet, calorie content and

Table 3: Agents for treating precancerous lesions and/or reducing cancer risk

Target Lesion/Organ	Treatment Agent	Target
Actinic keratosis	5-fluorouracil	DNA synthesis?
	Diclofenac sodium	Synthesis of prostaglandins?
	Imiquimod	Toll-like receptors 7-8, NFkβ?
	Ingenolmebutate	Mitochondria, Neutrophils?
	5-aminolevulinic acid + Photodynamic therapy	Damage to cellular machinery by ROS?
Bladder dysplasia	Bacillus Calmette-Guerin	Cellular immune machinery?
	Valrubicin	Not established
Breast cancer	Tamoxifen	Estrogen receptors
	Raloxifen	Estrogen receptors
	Exemestane	Aromatase enzyme (CYP450, CYPC19)
Cervical IEN and cancer vulvovaginal, anal IEN and cancer	HPV vaccines	
	Gardasil	HPV types 6, 11, 16, 18
	Cervarix	HPV types 16, 18
Colorectal polyps, adenomas and cancer	Aspirin	Cyclooxygenases
	Celecoxib	Cyclooxygenase-2
	Rofecoxib	Cyclooxygenase-2
	Sulindac	Ras pathway
	DFMO + Sulindac	Polyamine biosynthesis; Ras pathways
Esophageal dysplasia Preinvasive neoplastic lesion	Photofrin + PDT	Procaspase-3?
Hepatocellular carcinoma (HCC)	HBV vaccine	HBV
	Engerix-B	HBV-DNA
	Recombivax HB	HBV-surface antigen
Prostate	Finasteride	5-α-Reductase-type 1
	Dutasteride	5-α-Reductase-type 1+type 2
Skin (Melanoma)	Sunscreen (SPF 15+)	Blocking UVB (92%)

DMFO = Difluoromethylornithine, PDT=Photodynamic therapy, IEN=Intraepithelial neoplasia

physical activity (singly and in combination) on the standard agent mediated responses in humans. This is based on the extensive experimental and epidemiological observations on the protective effects of caloric restriction (Hursting *et al.*, 2010; 2013), diets rich in fruits and vegetables and moderate physical activity (World Cancer Research Fund/American Institute for Cancer Research, WCRF/AICR, 2007). Evidence on the protective effects of 'aspirin', 'statins' and 'metformin' in epidemiological studies further

suggest the role of carcinogen-mediated perturbation of common physiological pathways in cancer causation. Evidence generated may help in improving the planning, execution, monitoring and interpretation of outcomes from clinical trials. While consolidating on several of these aspects, efforts to search and exploit novel concepts such as 'synthetic lethality' (Davis and Wu, 2012) that has the potential to address both the issue of toxicity and efficacy in chemoprevention may also be undertaken.

This concept takes the advantage of the specific genetic changes in the precancerous cells to target them for destruction without harming the normal cells. Briefly, utilizing synthetic lethality approaches, chemo-preventive agents are delivered systemically, causing apoptosis selectively in premalignant cells without harming the normal cells, thus substantially decreasing toxicity. Furthermore, treatments are given for a short period of time, followed by intervals of drug-off periods. This method has been demonstrated to be effective in a mouse model of familial adenomatous polyposis (FAP) (Zhang *et al.*, 2010) as well as a mouse model of K-ras driven lung cancer (Huang *et al.*, 2011). Thus, targeting driver mutations with short-term Intermittent Therapy to Eliminate Premalignancy (SITEP) holds great promise for the future of chemoprevention.

Overall, clinical evaluation based on mechanistic studies, gene-environment interactions and complementary nutritional studies constitute the major thrust areas in chemoprevention research.

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CONFLICT OF INTEREST

The author claims no conflict of interest.

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